# The role of microRNAs and retinoid signaling during spinal cord regeneration in the adult newt.

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**Abstract**

The molecular events after spinal cord injury that lead to the establishment of a permissive environment and epimorphic regeneration remain unclear. Two molecular pathway regulators that may converge to create a spinal cord regeneration-permissive environment in the urodele are retinoic acid (RA) and microRNAs (miRNAs). Recent evidence suggests that RARβ-mediated signaling is necessary for tail and caudal spinal cord regeneration in the adult newt. MicroRNAs are attractive candidates as mediators of retinoid signaling during regeneration, as their pleiotropic effects are vital in situations where global changes in gene expression are required. Thus, the overall aim of this thesis was to determine if miRNAs are involved in tail and caudal spinal cord regeneration in the adult newt, and if they act as regulators and/or effectors of retinoid signaling during this process. I have demonstrated here, for the first time, that multiple miRNAs are dysregulated in response to spinal cord injury in the adult newt, as well as in response to inhibition of retinoid signaling. Two of these miRNAs, miR-133a and miR-1, appear to target RARβ2 transcripts both *in vivo* and *in vitro*. Inhibition of RA signaling via RARβ with a selective antagonist, LE135, alters the pattern of expression of these miRNAs, which leads to an inhibition of tail regeneration. These data are indicative of a negative feed back loop, albeit potentially an indirect one. I also aimed to examine which miRNAs are affected by inhibiting RA synthesis during regeneration, and provided a long list of miRNAs that are dysregulated. These data provide the foundation for future studies on the putative roles of these miRNAs, as well as their function in retinoid signaling. Overall, these studies provide the first evidence for a role for miRNAs as mediators of retinoid signaling during caudal spinal cord regeneration in any system.

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## Table of Contents

## List of Figures

## List of Tables

## List of Abbreviations

| | |
|---|---|
| ANOVA | Analysis of Variance |
| APL | Acute Promyelocytic Leukemia |
| ADH | Alcohol Dehydrogenase |
| BCA | Bicinchoninic Acid |
| BMP | Bone Morphogenic Proteins |
| BSA | Bovine Serum Albumin |
| BDNF | Brain-Derived Neurotrophic Factor |
| BCIP | 5-Bromo-4-Chloro-3-Indolyl Phosphate |
| BORIS | Brother of the Regulator of Imprinted Sites |
| CRABP | Cellular Retinoic Acid-Binding Protein |
| CNS | Central Nervous System |
| CSPGs | Chrondroitin Sulphate Proteoglycans |
| cDNA | Complementary Deoxyribonucleic Acid |
| dpa | Days Post Amputation |
| DEAB | Diethylaminobenzaldehyde |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic Acid |
| DMEM | Dulbecco's Modified Eagle's Medium |
| ECM | Extra Cellular Matrix |
| FGF | Fibroblast Growth Factor |
| ISH | *in situ* Hybridization |
| IFN | Interferon |

| | |
|---|---|
| IL-1β | Interleukin |
| LNA | Locked Nucleic Acid |
| mRNA | Messenger Ribonucleic Acid |
| MS-222 | Tricaine Methane Sulfonate |
| miRNA | MicroRNA |
| MIQE | Minimum Information for Publication of Quantitative Real Time PCR Experiments |
| Mps1 Kinase | Monopolar Spindle 1 Kinase |
| MAG | Myelin Associated Glycoprotein |
| MAI | Myelin Associated Inhibitors |
| NSC | Neural Stem Cells |
| NGF | Nerve Growth Factor |
| NT-3,4 | Neurotrophins 3 and 4 |
| NBT | Nitro Blue Tetrazolium |
| Nogo | Neurite Outgrowth Inhibitor |
| NgR | Nogo Receptor |
| NMLF | Nucleus Medial Longitudinal Fasciculus |
| OMgp | Oligodendrocyte-Myelin glycoprotein |
| OPC | Oligodendrocyte Precursor Cells |
| Pax7 | Paired Box Protein 7 |
| PNS | Peripheral Nervous System |
| PBS | Phosphate Buffered Saline |
| PTEN | Phosphatase and Tensin Homolog |
| pa | Post Amputation |

| | |
|---|---|
| PC3 | Human Prostate Cancer Cells |
| PML | Promyelocytic Leukemia gene |
| RhoA | Ras Homolog A |
| RALDH | Retinaldehyde Dehydrogenase |
| RA | Retinoic Acid |
| RAR | Retinoic Acid Receptor |
| RARE | Retinoic Acid Response Element |
| RXR | Retinoid X Receptor |
| RBP | Retinol Binding Protein |
| RDH | Retinol Dehydrogenase |
| RT-q-PCR | Reverse Transcriptase Quantitative Polymerase Chain Reaction |
| RNA | Ribonucleic Acid |
| RNase | Ribonuclease |
| RISC | RNA Induced Silencing Complex |
| Shh | Sonic Hedgehog |
| SC | Spinal Cord |
| SOD1 | Superoxide Dismutase 1 |
| TNF | Tumor Necrosis Factor |
| UTR | Untranslated Region |

# CHAPTER ONE:

## General Introduction and Literature Review

## 1.01 General Introduction

Regeneration is a phenomenon that has fascinated scientists for centuries. It is defined as an organism's ability to replace a lost structure, which can be achieved through several different processes including stem cell compensation, morphallaxis or epimorphic regeneration (Gilbert, 2000). Epimorphic regeneration involves the replacement of lost structures through blastema formation and proliferation of dedifferentiated cells, while morphallactic regeneration is simply the reorganization of the remaining tissue (Gilbert, 2000). The ability to regenerate varies widely across the animal kingdom, for example planaria are capable of regenerating almost an entire organism from a fraction of the original adult (Ermakova et al., 2009; Romero and Bueno, 2001). However this degree of regenerative capacity is rare, most mammalian species have a very limited capacity to regenerate lost structures in response to injury, particularly after reaching adulthood.

Urodele amphibians are unique in that they are the only tetrapod vertebrates capable of regenerating lost structures into adulthood, including limbs, heart tissue, lens, brain, tail and spinal cord (Chernoff et al., 2003; Lee-Liu et al., 2013). Understanding the mechanisms and signaling pathways that contribute to an organism's regenerative capacity is a major area of research, and could eventually lead to innovative approaches for injury treatments. The mechanisms that allow for tail and spinal cord regeneration in the newt remain poorly understood. It has been documented that retinoid signaling is vital for this process, and is specifically regulated through its ligand-activated receptor, retinoic acid receptor β (RARβ) (Carter et al., 2011; Dmetrichuk et al., 2005). However the upstream regulators of this signaling pathway as well as its downstream target genes remain unclear.

MicroRNAs are attractive candidates as effectors of retinoid signaling. They are small, non-coding RNAs that post-transcriptionally regulate gene expression. MicroRNAs are pleiotropic, meaning that they can have multiple targets, which makes them useful in situations where rapid changes in gene expression are required. This research examined the expression, localization and function of several microRNAs as potential effectors of retinoid signaling during caudal tail regeneration in the adult newt. Of particular interest were the microRNAs miR-1 and miR-133a, which were found to target RARβ, and appear to maintain its low levels of expression in tail and spinal cord tissue prior to injury. In response to a spinal cord injury, their downregulation is required and maintained by the upregulation of RARβ for functional tail regeneration. These data represent the first to examine microRNAs involvement in retinoid signaling during spinal cord repair in any species.

**1.02 Spinal Cord Injury and the Regenerative Capacity of Mammals**

Spinal cord injury (SCI) is a major health burden in humans since recovery from an injury to the central nervous system (CNS) is generally poor and can lead to permanent deficits such as paralysis. The World Health Organization estimates that more than 500,000 people suffer from an SCI each year (WHO, 2013). The majority of SCIs are the result of an accident and thus the type of injury is compression on the spinal cord, but the primary injury can also be the result of a stab lesion or blunt impact. This initial injury is characterized by immediate tissue damage, disruption of the meninges, hemorrhage, ischemia and wide spread cell death of glia and neurons (Anthony and Couch, 2014; Donnelly and Popovich, 2008; Harty et al., 2003; Zhou et al., 2014). While the primary trauma results in necrosis of tissue surrounding the site of injury, a

subsequent secondary response ensues, which leads to expansion of the injury site. This leads to further tissue death, as well as the establishment of both chemical and physical barriers that inhibit axonal regeneration (Zhou et al., 2014). This secondary injury response consists of inflammation, formation of the glial scar and the production of white matter inhibitors.

*Inflammation after SCI*

An immediate consequence of a SCI, and the beginning of secondary trauma, is the ensuing inflammatory response, which although in some ways is beneficial, can persist for years after the initial injury. The inflammatory response is evoked by the release of inflammatory cytokines and chemokines such as tumor necrosis factor α (TNF-α), interferon γ (IFN-γ) and interleukin β (IL-1β) from injured neurons and glia immediately after the injury (Donnelly and Popovich, 2008; Zhou et al., 2014). The first immune cells to migrate to the site of injury within the first few hours are neutrophils, which are circulating leukocytes that enter the lesion via chemotaxis. In the lesion, neutrophils release neurotoxic enzymes, free radicals and proteases that lead to further neuronal toxicity (Donnelly and Popovich, 2008; Zhou et al., 2014). Within the first week, lymphocytes infiltrate the site of injury, where reactive T cells mount a response against myelin and further exacerbate neuronal pathology (Donnelly and Popovich, 2008). The activation of T cells can lead to a parallel activation of B cells and thus antibody production, which can result in systemic neurodegenerative pathologies but also the beneficial production of neurotrophins. From 7 days post injury onward, macrophages infiltrate the lesion, and like leukocytes, they can be both beneficial as well as damaging. Activated macrophages and resident microglia release inhibitory cytokines, free radicals,

proteases and neurotoxic enzymes, and are also adept at killing surrounding healthy glia and neurons, actually increasing axonal dieback (Cregg et al., 2014; Donnelly and Popovich, 2008; Zhou et al., 2014). While for the most part, these effects of activated macrophages are inhibitory towards functional healing, they are also involved in neuroprotection because they actively remove the debris of dead cells from the site of the lesion, and in some cases release neurotrophic factors as well as modulating excitotoxicity. Unfortunately, another role of macrophages in secondary trauma after SCI is to promote migration of resident astrocytes to the lesion, which ultimately leads to astrogliosis (Fig. 1.1) (Fitch et al., 1999).

*Formation of the Glial Scar*

During astrogliosis, these resident astrocytes migrate not to the center of the lesion, but rather to the outer edges of the lesion, where secondary cell death occurs and they are activated and form the glial scar (Cregg et al., 2014; de Rivero Vaccari et al., 2012; Sahni et al., 2010; Shearer et al., 2003; Silver and Miller, 2004). Around the periphery of the scar, these astrocytes proliferate and become hypertrophic, increasing in size as well as upregulating expression of glial fibrillary acidic protein (GFAP), nestin and vimentin. These hypertrophic astrocytes come together to form a mesh like layer with their long filamentous processes intertwined, forming the glial scar (Cregg et al., 2014; Silver and Miller, 2004). This layer of astrocytes acts not only as a physical barrier impeding axonal outgrowth, but also produces proteoglycans such as chrondroitin sulphate proteoglycans (CSPGs), which are potent inhibitors of axonal regeneration. Thus, even those injured axons that escape the initial trauma and are capable of regrowth encounter the glial scar and cannot overcome these barriers, and develop dystrophic end

bulbs which can persist for decades (Cregg et al., 2014). Furthermore, after dieback dystrophic axons become closely associated with NG2+ oligodendrocytes precursor cells, and actually form synaptic-like connections with these cells (Filous et al., 2014). This association prevents axonal regrowth and represents another obstacle that needs to be overcome for recovery from a SCI. When the scar is mature and fully formed, it has two very distinct components, the penumbra, which is the layer of hypertrophic astrocytes around the perimeter, and the inner lesion core that consists of oligodendrocyte precursor cells (OPCs), ependymal cells, phagocytic macrophages, meningeal cells and pericytes.

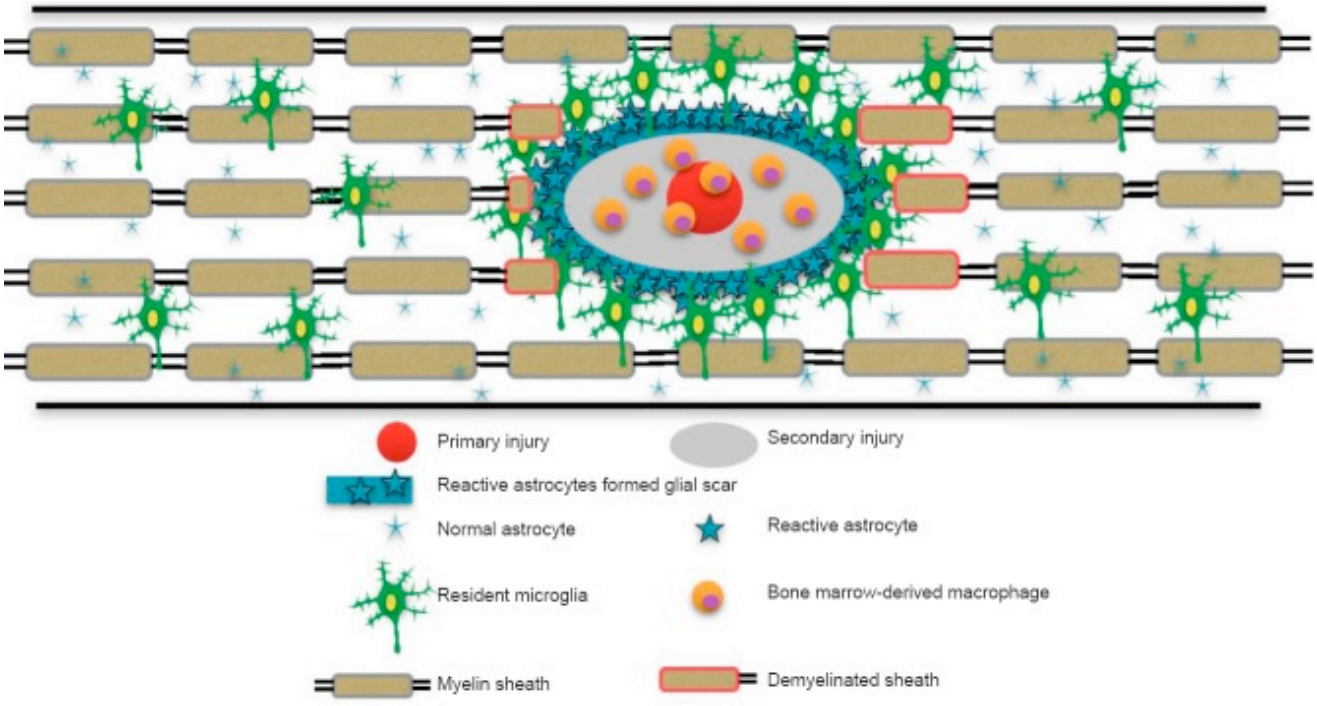


**Figure 1.1.** The site of the initial injury to the spinal cord (red) is much smaller than the cavitation and cell death caused by the secondary injury (grey) due to inflammation and glial scarring after SCI (Taken from Zhou et al., 2014- no copyright permission required).

*Myelin-Associated Inhibitors*

Inflammation and the formation of the glial scar together form an extremely inhibitory environment for axonal regrowth. Another contribution to this formation of an inhibitory environment comes from CNS myelin-associated inhibitors (MAIs), which are released with the breakdown of white matter during the SCI (Lang et al., 2014; Xie and Zheng, 2008). The three most common inhibitors are myelin-associated glycoprotein (MAG), neurite outgrowth inhibitor (Nogo) and oligodendrocyte-myelin glycoprotein (OMgp) (Lang et al., 2014). Their receptors can be found on the surface of neurons, with the most common shared receptor being the Nogo receptor (NgR). Ligand-bound-NgR inhibits neuronal growth through the activation of Rho kinase. This ultimately causes growth cone collapse through a negative modification of the actin cytoskeleton (Fujita and Yamashita, 2014; Xie and Zheng, 2008).

*Intrinsic Capacity for Axonal Regrowth*

Extrinsic inhibitors and glial scarring are not the sole reason for lack of axonal regrowth in the mammalian model; after development there is also a decrease in the number of growth promoting axonal guidance molecules such as neurotrophic factors (Giger et al., 2010). These factors include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT-3, NT-4). With the exception of areas that maintain a level of neural plasticity, such as the PNS and certain areas of the brain, these growth promoting factors are not present in the adult mammalian CNS (Giger et al., 2010). A lack of regeneration may also reflect a decreased intrinsic capacity of the injured neuron to respond to permissive extracellular cues (Muramatsu et al., 2009). For example, gene expression that is required for cytoskeletal rearrangement

may not occur, which would lead to an inability for the axon to respond to extrinsic growth promoting cues (Muramatsu et al., 2009). Taken together, the cascade of events during the first weeks post SCI creates an extremely inhibitory environment for functional regrowth, and often results in an expansion of the original injury site due to cavitation with secondary cell death as well as scar formation.

## 1.03 Spinal Cord Regeneration in Urodeles and Other Regeneration-Competent Species

Unlike mammals, adult urodele amphibians have the ability to regenerate several different tissues, including, but not limited to, lens, heart, limb and spinal cord (Chernoff et al., 2003, 2002; Iten and Bryant, 1976; Lee-Liu et al., 2013; Zukor et al., 2011). The regenerative capacity of these tetrapods makes them ideal models to study the cellular and molecular mechanisms that result in a permissive environment for spinal cord regeneration, as factors that are present in the newt may be altered in the mammalian system, or could be unique to the newt and thus completely absent in the mammalian model system. There are two methods of studying spinal cord regeneration that are frequently used in the urodele, spinal cord transection and caudal tail amputation (Fig. 1.2) (Lee-Liu et al., 2013). The spinal cord in the adult newt surrounds a central canal, which is bordered by ependymoglial cells, gray matter which consists of dorsal and ventral horns, and white matter and thick layers of collagen produced by meningeal cells (Chernoff et al., 2003).

*The Transection Model*

Following a complete transection of the spinal cord, functional recovery, which is measured by the ability to swim, has been recorded as early as 4 weeks post injury in the

newt (Davis et al., 1990). Lesion of the spinal cord in the newt triggers an ependymal response, where reactive ependymal cells proliferate and migrate into the lesion. The ependymal cells reorganize into a characteristic mesenchyme, and form an ependymal bridge which seals the ends of the cord stump (Chernoff et al., 2003). Axons then regenerate across the lesion in close association with glial processes and meningeal cells, regenerating through ependymal processes, using the ependymal bridge as support to reach the other side of the lesion and reestablish functional connections (Zukor et al., 2011).

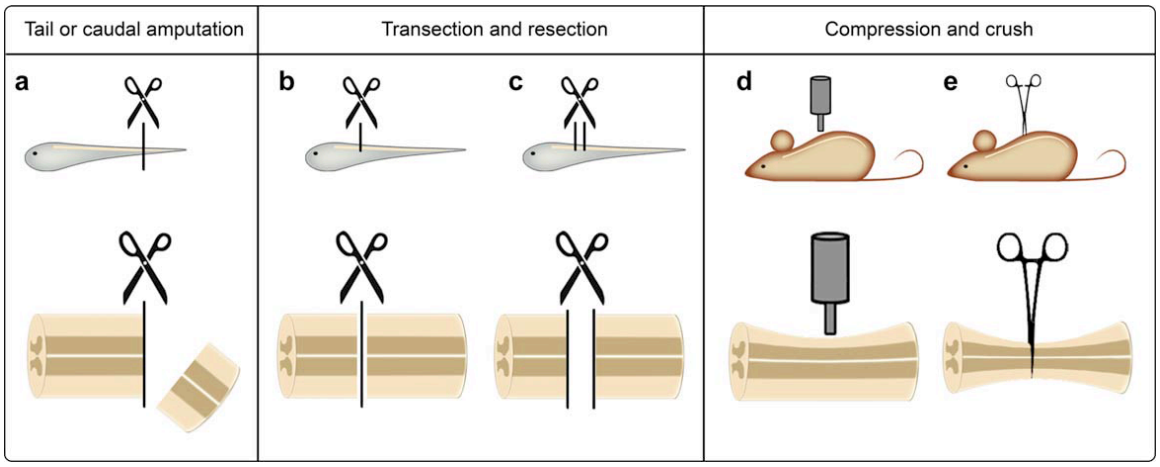


**Figure 1.2.** Different models used to study SCI, taken from Lee-Liu et al., (2013), copyright permission license #3646231145990. a) Caudal spinal cord regeneration following amputation of the tail of urodele amphibians as well as certain anuran tadpoles. b, c) Transection or resection of the spinal cord in urodele amphibians, which more closely resembles a crush injury, the most commonly used in the mammalian model system (d, e), as described above.

*The Caudal Tail Amputation Model*

The recovery from a complete transection of the spinal cord is simplistic compared to caudal tail regeneration, as the only area regenerating is the spinal cord and not surrounding tissue, and this model also bears a closer resemblance to SCI in mammals (Fig. 1.2) (Lee-Liu et al., 2013). However, the caudal tail amputation model is

more frequently used to study spinal cord injury in urodeles, in most cases due to the simplicity of the surgery compared to the transection models. This injury involves the complex regeneration and patterning of non-neural structures as well as the regrowth of the spinal cord (Chernoff et al., 2003; Diaz Quiroz and Echeverri, 2013; Lee-Liu et al., 2013). The hallmark of this type of epimorphic regeneration is the establishment of a wound epilthelium within the first 24 hours post amputation (Fig. 1.3) (Iten and Bryant, 1976), followed by the establishment of a highly proliferative mass of dedifferentiated cells which form the blastema (Tanaka and Weidinger, 2008). Contact between the spinal cord and wound epithelium is thought to induce the epimorphic regenerative response and blastema formation (Chernoff et al., 2003). The blastema also produces neurotrophic factors which stimulate neuronal regeneration in the tail (Bernard Bauduin et al., 2000). The blastema alone is capable of stimulating the regeneration of muscle and connective tissue, but is not sufficient to stimulate spinal cord regrowth, suggesting that non-blastema factors are involved in this process.

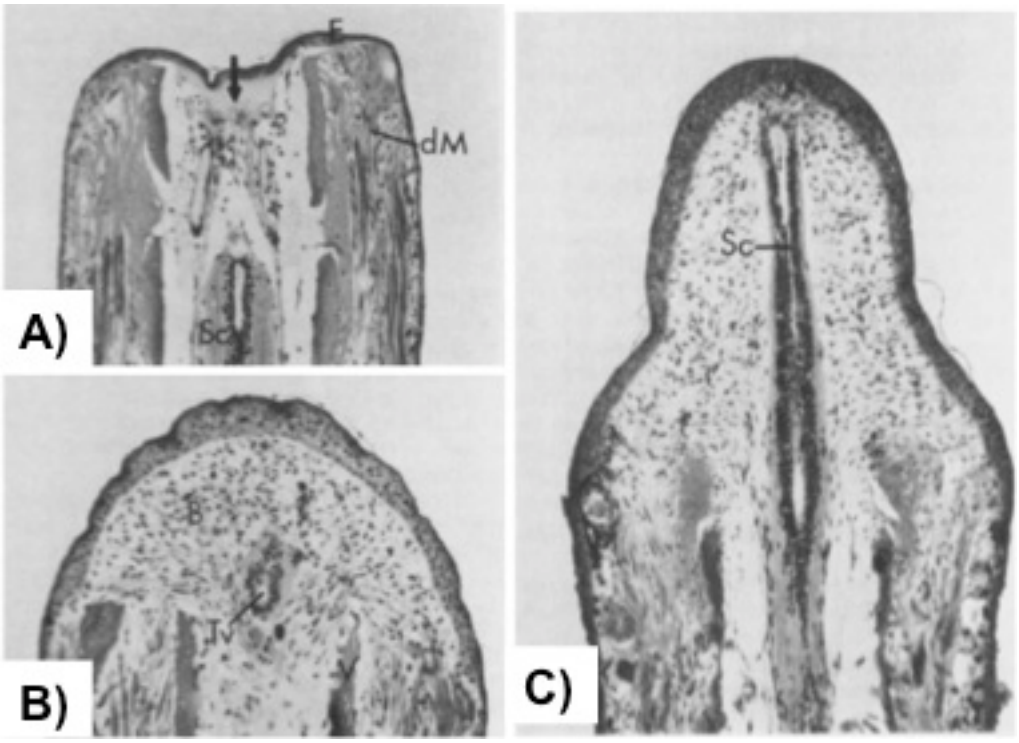


**Figure 1.3.** Sagital sections of the adult newt tail, *Notophthalmus viridescens*, during regeneration, taken from Iten and Bryant, (1976), copyright permission license #3646240103239. A) 2 day regenerate displaying a thickened epidermis (E) and degenerating muscle (dM) X 67. B) 10 day regenerate showing the terminal vesicle (Tv), blastema (B) and transected vertebra (V) X 71. C) 21 day regenerate displaying the regenerating spinal cord (SC) X 57.

*The Ependymal Response*

Similar to the transection model, caudal tail and spinal cord regeneration is highly dependent on an ependymal response (Chernoff et al., 2003). The ependymal cells rostral to the site of amputation proliferate, migrate caudally, and form a caudal hollow terminal vesicle which is continuous with the central canal of the spinal cord (Chernoff et al., 2003). Outgrowth occurs as a tubular extension within the regenerating tail, and these ependymal cells maintain this proliferative state along the length of the ependymal tube, while also maintaining their apical/basal polarity throughout regrowth (Chernoff et al., 2003). New neurons and glia are produced from the ependymal tube, and newly formed ependymal cells also give rise to other neural tissue in the regenerating cord that would

normally be derived from the neural crest (Chernoff et al., 2003). The ependymal tube also acts as a scaffold for regenerating axons that regrow from the tail stump into the extending regenerate. Thus, reinnervation of the regenerating tail occurs from new neurons that are born through neurogenesis during tail regeneration, as well as from existing neurons (Chernoff et al., 2003).

In both the spinal cord transection and caudal tail amputation injury models, there is an ependymal response, which is the critical step for spinal cord regeneration in both types of regeneration (Chernoff et al., 2003; Lee-Liu et al., 2013; Mchedlishvili et al., 2012). The ependymal bridge in transection model SCIs and ependymal tube in the tail regenerate not only act as a scaffold for axonal regrowth, but also give rise to new neurons, glia and other CNS and PNS tissue via an epithelial to mesenchyme transition (Chernoff et al., 2003; Echeverri and Tanaka, 2002; Mchedlishvili et al., 2012). These ependymal cells are morphologically similar to radial glia during development, and have been demonstrated to have neural stem cell characteristics (Lee-Liu et al., 2013; Panayiotou and Malas, 2013). What is interesting is that similar cells are present in the mammalian spinal cord and are thought to represent a population of neural stem cells in the spinal cord in these organisms (Barnabé-Heider et al., 2010). Indeed, mammalian ependymal cells initiate an "ependymal response" after SCI, with an increase in proliferation. However, their fate is somewhat restricted in that they only give rise to cells of different glial lineages (Barnabé-Heider et al., 2010).

*Factors Inhibitory to Regeneration in Mammals are Present in the Newt After SCI*

Considering that the environment of the lesion post-SCI in mammals is not conducive to regeneration, it would seem intuitive that the molecular environment in

urodeles would be completely different. Surprisingly, many of the factors that are inhibitory to regeneration in mammals such as CSPGs, MAIs and astrocytes are also present in the urodele (Hui et al., 2013; Zukor et al., 2011). Similar to mammals, there is an inflammatory response after spinal cord transection in the newt involving immune cells such as lymphocytes, monocytes and phagocytic macrophages (Zukor et al., 2011). In mammals, this inflammatory response is in some ways beneficial but ultimately causes more harm than good. Conversely, in the urodele the outcome of inflammation appears to be beneficial and not detrimental. Furthermore, ablation of macrophages in the axolotl limb during regeneration completely inhibited outgrowth (Godwin et al., 2013), suggesting that this inflammatory response is in fact a requirement for functional regrowth.

The outcome of the glial response to SCI is very different in mammals and urodeles. There are astrocytes present in the newt SCI lesion, however they do not become hypertrophic and do not form a glial scar (Zukor et al., 2011). Like mammals, there are also CSPGs and inhibitory extra cellular matrix (ECM) proteins, but they are not produced by astrocytes in response to injury and are not detrimental to axonal regeneration (Zukor et al., 2011). Finally, there are also MAIs released from damaged oligodendrocytes in the urodele during SCI similar to those observed in the mammal (Hui et al., 2013). Despite the fact that the NgR receptor for these MAIs is broadly expressed in the urodele spinal cord, inhibition of axonal outgrowth is not apparent. If the same types of inhibitory factors are present in the urodele as the mammal, how are they able to recover from an SCI? What factors or mechanisms post SCI allow for the establishment of a permissive environment for spinal cord regeneration in the urodele that are absent in

the mammal? The identification of the complex factors and signaling cascades that contribute to this permissive environment represent the focus of most current research on spinal cord regeneration in urodeles.

The cues that trigger the ependymal response and the establishment of a permissive environment in the urodele after injury remain elusive, however several factors have been implicated that may differ between the mammalian and urodele SCI models. Such factors include fibroblast growth factors (FGFs) and bone morphogenic proteins (BMPs) (Chernoff et al., 2003; Diaz Quiroz and Echeverri, 2013; Lee-Liu et al., 2013). In mammals, FGF signaling is associated with glial scar formation after SCI (Chernoff et al., 2003), while BMPs have been linked to inhibition of oligodendrocyte precursor differentiation (Lee-Liu et al., 2013). Conversely, both of these signaling molecules are associated with the ependymal response in urodeles (Diaz Quiroz and Echeverri, 2013; Lee-Liu et al., 2013). How each of these signaling factors work together to direct an ependymal response in one system, but have a different outcome in another is a current area of research, as is many other factors that contribute to regeneration and stemness of the ependymal cells in urodeles. However, for the remainder of this review the focus will be on the role played by retinoic acid, a signaling molecule important during development, maintenance and regeneration of the CNS.

**1.04 Retinoic Acid Synthesis and Signaling after SCI**

Retinoic acid (RA) is a small lipophilic molecule that is a biologically active metabolite of vitamin A and is crucial during development of the CNS. Vitamin A is extracted from the diet as retinyl esters from animal products or carotenoids from plants (Maden, 2007). Retinoids are stored as retinyl esters primarily in the liver, and when

needed, they are hydrolyzed to retinol in the liver for transport to target cells with retinol-binding protein 4 (RBP4), where retinol is taken up when RBP4 interacts with its membrane receptor STRA6 (Duester, 2008). Once in the cytoplasm, retinol is metabolized into all-*trans* RA (at-RA) in a two-step process. In the first step, RBP1 binds to retinol, which is then metabolized to retinaldehyde by retinol dehydrogenases (RDHs) (RDH1 and RDH10) and alcohol dehyrogenases (ADHs) (ADH1, ADH3 and ADH4) (Duester, 2008). The final step in RA synthesis occurs when retinaldehyde is oxidized to RA by several retinaldehyde dehydrogenases (RALDH1, 2 and 3). Newly synthesized RA can act either in a paracrine or autocrine manner. In its target cell, it enters the nucleus with the assistance of cellular retinoic acid binding protein (CRABP2). In the canonical pathway RA can bind to retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which would trigger the formation of an RAR/RXR heterodimer (Maden, 2007). There are three RAR and RXR subtypes (RARβ, RARγ, RARα) (RXRγ, RXRβ, RXRα), each of which has different isoforms. Once bound to RA, the heterodimer associates with a transcription complex that binds to a retinoic acid response element (RARE), leading to the activation or repression of the expression of its target gene (Fig. 1.4) (Duester, 2008; Maden, 2007).

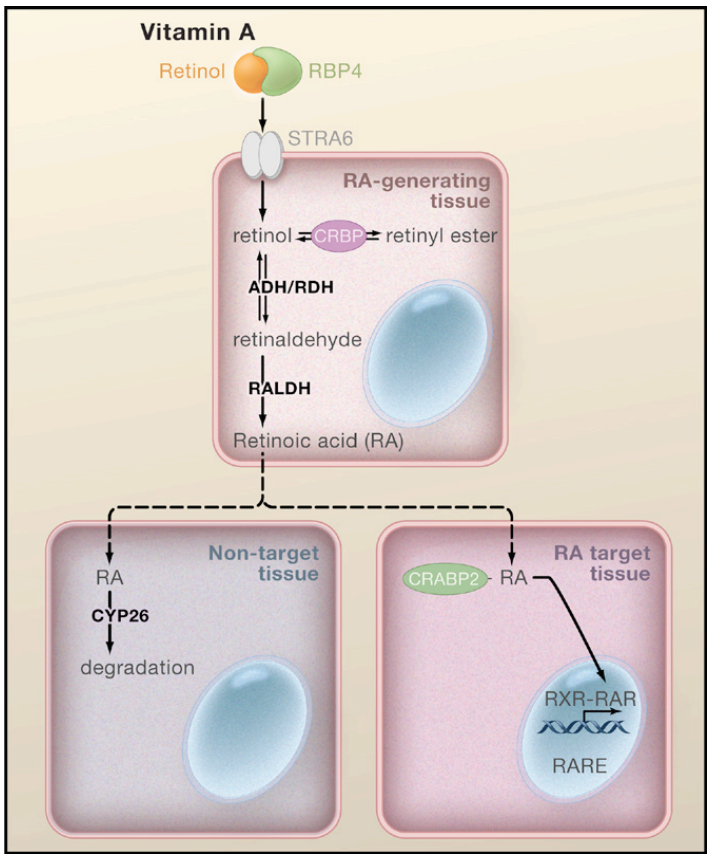


**Figure 1.4.** The canonical retinoic acid synthesis and signaling pathway. All-*trans* RA is synthesized through a two-step process, where it can then act on its own or a nearby cell. It enters the nucleus and binds to a RAR, which heterodimerizes with an RXR to activate or repress target gene expression, taken from Duester, (2008), copyright permissions not required.

This cascade represents the classic RA synthesis and signaling pathway. Parallel sequencing and bioinformatics with multiple cell types have suggested that there are thousands of genomic RAR binding sites (Rochel and Moras, 2014), however not all RA-regulated gene networks are associated with a RARE. There are several studies that suggest a non-canonical role for RA signaling, including interactions with intermediate transcription factors, receptor association with other regulatory proteins, or even non-genomic receptor actions (Balmer, 2002; Farrar et al., 2009). Furthermore, even within the canonical RA signaling pathway, potential pleiotropic effects on target expression are

possible, as there are multiple combinations of RAR and RXR isoforms, which when hetero or homodimerized, could associate with multiple targets (Maden, 2007).

*Retinoid Signaling in the Mammalian and Avian CNS*

It has long been established that RA is involved in the patterning, development, maintenance and regeneration of the CNS (Blum and Begemann, 2013; Duester, 2008; Maden, 2007). During development, RA acts as a morphogenic factor, and is involved in the patterning of the anterior/posterior and dorsal/ventral axes of the neural tube and neural plate. In the neural tube pathway, RA is synthesized by newly formed somites, and specifies the fate of sensory and motor neurons, and is also responsible later on in development for the organization of the hindbrain and anterior segment of the spinal cord (Duester, 2008; Maden, 2007). RA can be found throughout the developing CNS, but is particularly abundant in the developing spinal cord, where it may also act as a chemotactic factor (Maden et al., 1998). Indeed, RA is capable of stimulating directed neurite outgrowth from both embryonic mouse dorsal root ganglia (DRG) (Corcoran et al., 2000), and embryonic chick DRGs *in vitro* (Maden et al., 1998). RA also elicits neurite outgrowth from adult rat DRGs, and this outgrowth is accompanied by an upregulation of both RARβ2 and RALDH2 (Corcoran and Maden, 1999). Futhermore, deletion of *RARβ2* in mice significantly decreased the amount of neurite outgrowth of DRGs *in vitro* (So et al., 2006). These, and other data highlight the role of RARβ2 in modulating RA-stimulated neurite outgrowth in these cultures. For example, embryonic mouse DRGs express all three subtypes of RARs, but only DRGs that expressed RARβ2 were able to respond to RA treatment (Corcoran et al., 2000).

While RA is able to induce directed neurite outgrowth in the adult PNS, the same abilities do not transcend to the adult CNS. Similar to the PNS, embryonic spinal cord explants from the mouse cultured with exogenous RA exhibit neurite outgrowth, however adult spinal cord explants were unresponsive (Corcoran et al., 2002). It was speculated that perhaps expression of RARβ2 was lost in the adult mammalian CNS, and that this lack of neurite outgrowth in response to RA treatment was due to an intrinsic inability to transduce the RA signal. Indeed, when adult mouse spinal cords were transduced with a lentiviral vector expressing *RARβ2,* they exhibited neurite outgrowth in response to RA similar to that seen in the embryonic explants (Corcoran et al., 2002). These data suggest RARβ2 is a crucial transducer of the RA signal and its downregulation in the adult mammalian CNS may contribute to a lack of regeneration after SCI.

*Increased RARβ Expression is Consistent with Regeneration In Vivo*

Increasing expression of RARβ2 has been shown to increase neurite outgrowth in non-permissive environments for both PNS and CNS neurons *in vivo* (Wong et al., 2006; P K Yip et al., 2006). Adult rat DRGs that were transduced with a lentivirus expressing *RARβ2* exhibited neurite outgrowth *in vitro* and *in vivo*; specifically, they were able to regenerate past the inhibitory dorsal root entry zone to regain a level of both sensory and motor function (Wong et al., 2006). In a similar study, Yip et al., (2006) demonstrated that the same outcome was possible with neurons in the CNS. Corticospinal tract neurons transduced with the same lentiviral *RARβ2* vector were capable of crossing a spinal cord lesion, achieving a modest degree of locomotor function. Each of these studies provides support for the postulate that not only is RARβ2 a crucial transducer of RA for neuronal regeneration, but that it is lost in the adult mammalian CNS. However, more recently,

Agudo et al., (2010) provided evidence that RARβ2 expression, while downregulated in the adult mammalian spinal cord, is in fact expressed in both the cerebellum and cerebral cortex. When adult rat cerebellar neurons were cultured with an agonist of RARβ, CD2019, they were capable of growth even in the presence of the myelin associated glycoprotein (MAG), a potent inhibitor of neurite outgrowth. Moreover, this RARβ2 selective agonist was able to promote some functional recovery of the SCI in adult rats (Agudo et al., 2010).

Another study examined the role of Lingo-1 as a potential target of RARβ2 in the mouse SCI model. Lingo-1 is associated with NgR, and its expression is associated with the production of a non-permissive environment for axonal growth in the adult mammalian CNS (Puttagunta and Di Giovanni, 2011). It was found that RARβ2-mediated the downregulation of Lingo-1 post-SCI in these mice and resulted in a more favorable outcome with some functional recovery. These studies demonstrated that when RARβ2 is expressed in the adult mammal, it might contribute to the establishment of a permissive environment leading to axonal outgrowth and some recovery of function.

*Retinoic Acid Signaling and the Inflammatory Response to SCI*

Previous studies have also demonstrated that RA has anti-inflammatory capabilities. Systemic administration of RA immediately after SCI in the rat reduced the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα, and thus the overall amount of secondary damage due to inflammation (van Neerven et al., 2010). As previously discussed, it has been demonstrated that inhibitory factors such as MAIs and inflammation occur in the urodele (Hui et al., 2013; Zukor et al., 2010). Urodeles are capable of recovery from SCI, so RAs involvement in inflammation contributes to the

idea that a difference between adult mammals and urodele amphibians with respect to regenerative capacity could be the ability to transduce the RA signal.

*Retinoid Signaling and Regeneration in Urodele Amphibians*

The role of RA has been examined during both limb and spinal cord regeneration in the urodele. During limb regeneration, inhibition of RA synthesis blocks regeneration and leads to patterning abnormalities (Maden, 1998). RA is synthesized during limb regeneration in the apical epithelium (Monaghan and Maden, 2012), with overlapping expression of RARδ1 (Hill et al., 1993), a potential transducer of the RA signal during limb regeneration. During spinal cord regeneration in the urodele, RA does in fact appear to exert a stimulatory effect on neurite outgrowth in this model system that leads to complete functional recovery and morphological reconstitution. Adult newt spinal cord explants cultured with limb blastemas exhibited enhanced neurite outgrowth compared to explants cultured alone (Bauduin et al., 2000). Interestingly, limb blastemas pre-treated with citral (an RA synthesis inhibitor) had significantly reduced neurite outgrowth promoting activity (Prince and Carlone, 2003). These studies suggest that RA produced from limb blastemas is capable of promoting neurite outgrowth *in vitro*. In a study similar to those examining the effects of exogenous RA on embryonic mouse spinal cord explants, Hunter et al., (1991) demonstrated that RA treatment of adult axolotl spinal cord explants *in vitro* enhanced neurite outgrowth. Dmetrichuk et al., (2005) also demonstrated that RA is capable of acting as a chemotactic factor in the urodele as it is in the mammalian model. Beads soaked with RA and placed near spinal cord explants produced neurite outgrowth, and this outgrowth was directed towards the source of RA. Furthermore, this chemotactic effect of RA appeared to be mediated by RARβ, as

treatment with LE135, an RARβ-selective agonist completely abolished the trophic effects of exogenous RA treatment *in vitro*.

The previous data are suggestive of a role for RARβ-mediated retinoid signaling in the urodele after SCI. In support of this notion, Carter et al., (2011) examined the expression pattern of RARβ2 in the adult newt, and found that unlike in the adult mammal, RARβ2 is expressed throughout the spinal cord and brain. In response to injury, RARβ2 is upregulated by 7 days post amputation (dpa), and expression is localized to the ependymal tube in the regenerating tail. Inhibition of RARβ2 with the selective antagonist, LE135 led to an inhibition of tail regeneration and a lack of ependymal tube formation (Carter et al., 2011). These data are very interesting, as the ependymal response and formation of the ependymal tube is essential for functional spinal cord regeneration in the urodele after tail amputation. Thus, the apparent role of RARβ2 post SCI is consistent with what has been described in mammals and highlights the importance of RARβ2 for functional regeneration. Expression of RARβ as well as other RA signaling genes does occur in the ependymal cells surrounding the third ventricle in adult rats (Helfer et al., 2012). It is possible that expression of RARβ in the ependymal cells surrounding the spinal cord provides them with the stem cell like qualities observed in the urodele but not mammalian CNS. However, the precise targets of RARβ2 signaling during the ependymal response remain to be elucidated. Uncovering the regulators of RARβ2 expression, as well as its downstream targets could provide a clearer picture of the mechanisms that allow for regeneration in the urodele after SCI.

### 1.05 MicroRNA Biogenesis and Function During Regeneration

Although the role of RARβ has been examined in detail in both the mammalian and urodele SCI models, very little research has examined the upstream factors or signaling pathways that activate RARβ2, or the downstream targets of RARβ2-mediated RA signaling during regeneration. MicroRNAs (miRNAs) are attractive candidates as effectors of retinoid signaling during recovery from SCI. miRNAs are an abundant class of small, endogenous non-coding RNAs that are approximately 22 nucleotides in length and can be found in most somatic cells (Ha and Kim, 2014; Kim et al., 2009; Kosik, 2006; Thatcher and Patton, 2010). They are very highly conserved among distantly related species, and regulate gene expression post-transcriptionally. It is thought that more than 60% of protein coding genes are under the control of miRNAs (Ha and Kim, 2014; Liu et al., 2009). miRNAs are involved in many biological processes including developmental timing, stem cell differentiation and signal transduction (Corbin et al., 2009; Ha and Kim, 2014; Han et al., 2011; Hancock et al., 2014a). Their expression is very tightly regulated, so it is no surprise that miRNA dysregulation is often associated with cancer and other diseases (Ha and Kim, 2014). Many miRNAs are encoded within multiple loci, and have very similar sequences. However in order to be considered a part of a miRNA family, each miRNA must have the same "seed" sequence (nucleotides 2-8). Sister miRNAs can have very different localizations and thus functions, but for the most part are capable of acting redundantly on target mRNAs due to these identical seed regions (Ha and Kim, 2014). There are 34 families of miRNAs that are conserved from *C. elegans* to humans, and 196 families conserved among all mammals. Although nomenclature varies, miRNAs from different loci end with a number (miR-133a-1, miR-

133a-2), while those from each precursor strand have a corresponding strand ID (5'= miR-133a-5p, 3'= miR-133a-3p) (Ha and Kim, 2014).

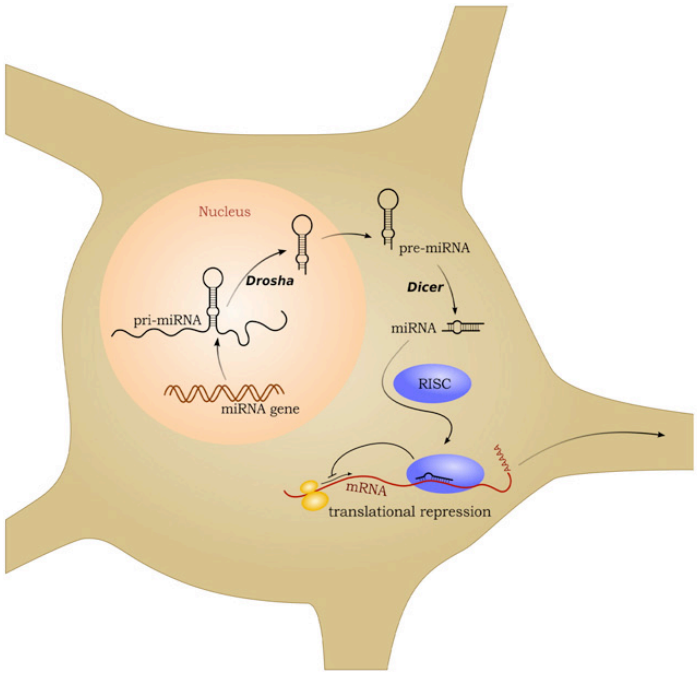


**Figure 1.5.** MicroRNA biogenesis and function. MicroRNAs are transcribed in the nucleus then undergo two processing steps, one in the nucleus one in the cytoplasm, to form the mature miRNA which is incorporated into the RISC complex to target mRNA for translational repression. Taken from Fiore et al., (2008), copyright permission license # 3646451404901.

*Transcriptional Regulation and Processing*

Generally, miRNAs are encoded within the introns of both coding and non-coding transcripts, however there are examples where miRNAs are encoded by exonic regions as well (Ha and Kim, 2014). Many miRNA loci are often located within close proximity to each other within poly-cistronic transcription units, and these clusters are often co-regulated, however epigenetic and post-transcriptional modifications can also influence miRNA expression. Transcription of miRNAs is mediated by RNA polymerase II and its associated factors, which generates a hairpin primary miRNA (pri-miRNA) transcript that is over a kilobase long (Fig. 1.5) (Ha and Kim, 2014; Kim et al., 2009; Kosik, 2006;

Thatcher and Patton, 2010). Processing of this pri-miRNA to its mature form occurs in several steps. The hairpin of the pri-miRNA contains the mature miRNA sequence, and has a long tail at the 3' and 5' ends, and enters a microprocessing unit containing the nuclear ribonuclease (RNase III) Drosha and its cofactor DGCR8. The stemloop is recognized by DGCR8, which triggers Drosha to asymmetrically cut the stemloop, liberating a stemloop with a 5' phosphorylated end and a 3' 2 nucleotide overhang, the double stranded pre-miRNA (Kim et al., 2009; Thatcher and Patton, 2010). The pre-miRNA is then transported from the nucleus via the nuclear transport receptor Exportin 5, where it undergoes its second processing step. The cytoplasmic RNase III, Dicer, recognizes the 3' overhang of the pre-miRNA. The PAZ domain of Dicer contains two pockets, one interacts with the phosphorylated end of the pre-miRNA, the other with the 3' overhang (Ha and Kim, 2014). Dicer then cleaves the pre-miRNA, which generates a mature miRNA duplex. This duplex is unwound when it associates with a member of the Argonaut protein family. Generally the less stable strand is kept while the other is discarded for degradation (Kim et al., 2009). Together with these Argonaut proteins, the mature miRNA is then incorporated into the RNA induced silencing complex (RISC). The mature miRNA recognizes and binds to the 3' untranslated region (UTR) of its target mRNA, through Watson-Crick basepairing, with the key recognition occurring through the seed region (Saugstad, 2010; Valinezhad Orang et al., 2014). A perfect match of the miRNA to its target results in Argonaut-catalyzed cleavage, while central mis-matches result in translational repression, deadenylation and mRNA decay. Since it is only vital that the seed region of a miRNA binds to its target mRNA, it is not only possible that each miRNA could have many target mRNAs, but also that any given mRNA could be

regulated by multiple miRNAs, indicating that miRNAs could potentially regulate whole families of genes (Bhalala et al., 2013).

*MicroRNAs and SCI in Mammals*

The pleiotropic effects of miRNAs make them ideal candidates for situations where global changes in gene expression need to occur rapidly, such as after an SCI. Indeed, there have been several studies that have examined miRNA expression in response to SCI using microarray-based analyses (Liu et al., 2009; Nakanishi et al., 2010; Nieto-Diaz et al., 2014; Strickland et al., 2011; Yunta et al., 2012). These have revealed hundreds of miRNAs that are dysregulated in response to injury. Liu et al., (2009) identified a large number of miRNAs that were differentially expressed in the rat in response to SCI, and predicted that many of these miRNAs may have roles in inflammation, apoptosis and oxidative stress that contribute to secondary damage. In a similar study, Nakanishi et al., (2010) used a microarray to identify differentially expressed miRNAs after SCI in the mouse, and focused on the miRNAs, miR-124a and miR-223. They found that miR-124a is highly expressed in the uninjured spinal cord, but downregulated in response to injury (Yunta et al., 2012), while miR-223 expression increased in response to injury and was localized to the lesion site. miR-124a is thought to be a neural-specific miRNA, as it is highly expressed during neurogenesis, but in this case is likely downregulated in association with cell death in the nervous system. Conversely, miR-223 has been associated with inflammation, hence its upregulation in the lesion core. A microarray by Strickland et al., (2011) confirmed the downregulation of miR-124 as well as miR-1 in response to SCI, and also found an increase in miR-21,

which was localized in the same cells as SOX2, nestin and REST, suggestive of a stem cell niche.

While each of these studies uncovered a plethora of miRNAs whose expression changes due to a SCI, the majority of their functions in response to injury remain unknown. There are, however, a few studies that have begun to unravel some of the functions of identified miRNAs during secondary trauma. While several miRNAs are associated with secondary damage, it is possible that understanding their function could lead to treatments for SCI in the mammal. miR-223 has been implicated at various stages of inflammation, but importantly is highly expressed in neutrophils that contribute to the early inflammatory response and contribute to secondary damage post SCI (Izumi et al., 2011). Both miR-21 and miR-125b have been implicated in astrogliosis and the formation of the glial scar (Bhalala et al., 2012; Pogue et al., 2010). Downregulation of miR-21a has been associated with neural protection post SCI via the upregulation of its target, neurogenin (Jee et al., 2012).

miRNAs have also been shown to mediate the differentiation of both neural stem cells and oligodendrocytes (Zhao et al., 2010). For example, the miRNAs let-7, miR-9 and miR-124 have been shown to promote neural stem cell differentiation, while miR-25, miR-134 and miR-137 induce neural stem cell proliferation (Meza-Sosa et al., 2014). Expression of miR-132 is also associated with axonal extension in mouse DRGs during development, where its translational control over *Rasa1* promotes outgrowth (Hancock et al., 2014b). This study along with others have identified multiple miRNAs that were present in extending axons (Iyer et al., 2014), which suggests a potential role for miRNAs in the promotion or inhibition of axonal regrowth post SCI. Thus, it is evident that

miRNAs are likely involved in virtually every step of secondary damage that occurs after a SCI in mammals. Understanding their role, as well as how their function and targets may differ in regenerative-competent vertebrates such as the zebrafish and newt could provide insight on how to combat these pathologies.

*MicroRNAs in Regeneration-Competent Species*

There have been multiple studies that examine the role of miRNAs during appendage and organ regeneration in regeneration-competent species (Nakamura et al., 2010; Thatcher et al., 2008; Tsonis et al., 2007; Witman et al., 2013; Yin et al., 2008), but relatively few have focused on tail and spinal cord regeneration (Sehm et al., 2009; Yu et al., 2011). The let-7 family of miRNAs has been implicated during lens regeneration in the newt (Tsonis et al., 2007). In particular, let-7b downregulation is necessary for stem cell proliferation during lens regrowth (Nakamura et al., 2010). Cardiac regeneration has been examined in both the newt and zebrafish. Witman et al., (2013) used a microarray to identify miRNAs that were expressed at various stages of heart regeneration in the newt, and found that miR-128 was elevated and targeted *Islet1*. In a similar study, Yin et al., (2012) demonstrated that miR-133 was downregulated during zebrafish cardiac regeneration, and experimental induction of this miRNA inhibited functional recovery. While these studies do not focus on tail or spinal cord regeneration, they are interesting as at least some of the processes are common to all types of epimorphic regeneration, including those associated with injury to the tail or spinal cord. These include inflammation, stem cell maintenance and anti-apoptotic functions. Of particular interest are studies that have examined limb/fin regeneration, as in this case there is formation of a blastema and specialized epithelium, as there is in tail regeneration in newts and

salamanders. Yin et al., (2008) and Thatcher et al., (2008) both examined miRNAs that were vital for caudal fin regeneration in zebrafish. miRNA 133a was found to be high in uninjured fins, but significantly downregulated in response to fin amputation (Yin et al., 2008). It was hypothesized that perhaps miR-133a acts as a "regenerative break", as it targets *mps1*, a kinase that regulates proliferation in the FGF signaling pathway. Thus levels of miR-133a are high when proliferation is not needed but downregulated to allow for re-entry into the cell cycle in response to injury. Similarly, Thatcher et al., (2008) found that miR-203 is downregulated in response to injury and targets *lef1* in the Wnt signaling pathway. The authors speculated that miR-203 and miR-133a are both downregulated in response to injury to allow for the coordinated activation of these pro-regenerative pathways. These data suggest that it may not necessarily be miRNA upregulation in response to injury that is important for regeneration, but in some cases their downregulation is vital to allow for the expression of genes required for functional recovery. Conversely, Holman et al., (2012) found that miR-21 is upregulated in the axolotl during limb regeneration, and was found to target *Jagged* to promote proliferation of mesenchyme cells. Interestingly, this miRNA is often associated with several cancers in humans, including breast, prostate, colon, pancreatic and esophageal. This fact is not surprising as processes required for the development of tumors, such as loss of cell cycle control or apoptotic factors, are similar to those required for the stages of regeneration.

*MicroRNAs and Spinal Cord Regeneration*

Understanding the roles of miRNAs during tail and spinal cord regeneration in regenerative competent species such as the urodele and zebrafish are just starting to be examined, and relatively few studies have been published thus far. Sehm et al., (2009)

used a microarray to examine which miRNAs were differentially expressed in response to tail amputation in the axolotl. Of particular interest was miR-196, which was highly upregulated, and inhibition of this miRNA inhibited tail regeneration. It was found to be an upstream effector of *BMP4* and *Pax7*, thus it is involved in the patterning of the regenerating spinal cord. As described above, there are two methods of studying SCI in regenerative competent species, tail amputation or transection. Yu et al., (2011) examined miRNA expression after a complete transection of the spinal cord near the brain stem in zebrafish. Upregulation of miR-133b was observed, and inhibition of this miRNA also inhibited locomotor recovery. In this study, miR-133b was found to target *RhoA*, a GTPase protein that is activated by MAIs binding to the Nogo receptor, which was described above. RhoA is a potent inhibitor of axonal outgrowth, and thus the upregulation of this miRNA is necessary to promote functional regeneration of the spinal cord. In a similar study, Diaz Quiroz et al., (2014) used microarray data to identify miRNAs that were differentially expressed after a complete transection of the spinal cord in the axolotl compared to rats. Of particular interest was miR-125b, which is highly expressed in the axolotls radial glial cells, but downregulated in response to SCI. miR-125b was found to target Sema4D, a transmembrane axonal repulsion cue, and it is likely that this miRNA is involved in lack of glial scar formation as well as promotion of axonal regeneration (Diaz Quiroz et al., 2014). Again, these data are intriguing as it is clear that miRNAs are vital during epimorphic regeneration. However, to date there has been no research that examined miRNA expression in the newt after tail amputation or spinal cord transection, and none in any system (mammalian or regeneration-competent), that examine miRNAs as effectors of retinoid signaling in response to SCI.

## 1.06 Objectives

The main aim of this thesis was to uncover the potential role of miRNAs during tail and caudal spinal cord regeneration in the adult newt, and to determine whether they may either regulate and/or be regulated by retinoid signaling pathways during this process. There is evidence that miRNAs may be involved after an SCI in mammals, as well as regeneration of other structures in urodeles and fish. Furthermore, retinoid signaling has been shown to play a vital role in not only the ability of urodeles to recover from an SCI, but also the lack of recovery observed in mammals. Thus I hypothesized that miRNAs are involved during tail and spinal cord regeneration in newts, and that some of these miRNAs may be acting as up or downstream effectors of retinoid signaling during this process. Our model system was the eastern spotted newt, *Notophthalmus viridescens*, which is capable of regenerating limb, heart, lens, skin, tail and spinal cord tissue (Fig. 1.5).

**Figure 1.6.** The eastern spotted newt, *Notophthalmus viridescens*, which was used as the model system for every study in this thesis.

The first specific aim of this research was to determine which, if any, miRNAs are involved in spinal cord regeneration in the adult newt. Quantitative polymerase chain reaction (q-PCR) was used to amplify target miRNAs (miR-133a, miR-132, miR-124a and miR-203), and to observe their temporal pattern of expression during early tail regeneration. To determine in which tissues miRNAs 133a and 203 were expressed, *in situ* hybridization was used on tissue sections obtained from regenerates at early stages of regeneration. Finally, the function of these miRNAs was examined using the *in vivo* injection and electroporation of miRNA mimics and inhibitors.

My next objective was to determine which, if any miRNAs were specifically acting downstream of retinoid signaling. This was accomplished using a microarray

analysis comparing the expression of miRNAs in caudal tail regenerates to those in regenerates in which RARβ was inhibited by a selective antagonist, LE135. From this list, selected miRNAs were further examined to determine the spatial and temporal pattern of expression, as well as function, using the above techniques.

Inhibition of RARβ only examined miRNAs that were effectors of retinoid signaling, thus my final objective was to uncover other miRNAs that could be acting as effectors at any point during retinoid synthesis and signaling. To accomplish this, RA synthesis was inhibited using an inhibitor of RALDH2, diethylaminobenzaldehyde (DEAB), and again a microarray was used to identify miRNAs involved in this pathway. A selection of these miRNAs was then quantified temporally using q-PCR to lay the groundwork for future studies on their function. Overall, this thesis provides data for the first study of miRNA involvement in retinoid signaling during spinal cord repair in any model system.

# CHAPTER TWO:

## RARβ2 expression is induced by the down-regulation of microRNA 133a during caudal spinal cord regeneration in the adult newt.

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## 2.01 Abstract

Adult urodele amphibians represent unique model organisms to study spinal cord regeneration. Trauma to the spinal cord induces an ependymal response, activating multipotent neural stem cells which contribute to the redifferentiation of both glia and neurons in the regenerate. The molecular events underlying this ependymal response are not completely understood, but likely involve coordinated global changes in gene expression. MicroRNAs (miRNAs) and retinoid signaling are postulated to orchestrate these patterns of gene expression in response to trauma. Our objectives were to determine the roles played by some miRNAs as potential regulators of retinoid signaling in this process. We found that the expression levels of miRNAs 133a, 203 and 124a are dysregulated during the first 21 days post amputation (dpa). Interestingly, these miRNAs are expressed primarily within the ependymoglia. We have shown *in vitro*, that a miR-133a mimic targets the 3' UTR of the newt RARβ transcript. Importantly, upregulation of this mimic *in vivo* led to a significant decline in RARβ protein at 14 dpa and inhibited regeneration. These data are the first to link miRNAs and retinoid signaling during spinal cord regeneration and provide support for miR-133a as an upstream regulator of RARβ expression in this process.

## 2.02 Introduction

Unlike adult mammals, the adult red spotted newt, *N. viridescens*, is capable of regenerating several types of tissue after injury, including the spinal cord (SC) (Chernoff et al., 2003; Diaz Quiroz and Echeverri, 2013; Mchedlishvili et al., 2012; Zukor et al., 2011). After complete transection of the tail, a population of neural stem cells, the ependymoglial cells, from the damaged caudal SC, proliferate and extend caudally to initiate the regeneration of the missing tissues. This "ependymal response" is coincident with the formation of a regeneration blastema, composed of stem-like cells that have either de-differentiated from local stump tissues or have been recruited to the damaged area (Chernoff et al., 2003; Lee-Liu et al., 2013). The ependymoglial cells will ultimately contribute to the redifferentiation of spinal cord neurons and glial cells.

The molecular events that underlie this process are not well understood and likely involve global changes in gene expression. One factor that may be critical in this process is a biologically active metabolite of vitamin A, all-trans retinoic acid (RA) (Blum and Begemann, 2013; Maden, 2007). RA is known to modulate the growth and differentiation of many cell types and is an important morphogenetic molecule in the embryo (Duester, 2008). It is abundant in the central nervous system (CNS) of vertebrates (Corcoran et al. 2002; Malcolm Maden and Hind 2003), particularly in the developing spinal cord (Maden et al., 1998), and is required for normal development of the CNS (Maden, 2007; Zhao et al., 2008). The actions of RA on target cells are mediated by ligand activated nuclear transcription factors, the retinoic acid (RAR) and retinoid X (RXR) receptors, ultimately inducing a change in gene activity. At least four isoforms of RARs have been identified in regenerating newt tissues (Maden, 1996). It has been hypothesized that one of many

factors contributing to the inability of adult mammalian spinal cord neurons to regenerate is a post-embryonic inactivation of one or more of the RARs, particularly RARβ (Corcoran et al. 2002). In support of this hypothesis, it was shown that a specific RARβ agonist could overcome the inhibitory effects of myelin associated glycoprotein (MAG) on neurite outgrowth *in vitro* and could promote functional recovery in a rat spinal cord injury model (Agudo et al., 2010).

In adult spinal cord tissue of a regeneration-competent species such as the newt, RARβ expression is maintained and significantly increased in ependymoglial cells of the spinal cord tissue within the first 7 days following tail resection (Carter et al., 2011). Moreover, *in vivo* inhibition of retinoid signaling by administration of a selective antagonist for RARβ significantly inhibits spinal cord regeneration (Carter et al., 2011).

The multiple pathways either regulating or regulated by RA signaling during regeneration of the spinal cord in the newt remain to be elucidated. MicroRNAs (miRNAs), a class of small non-coding RNAs, are attractive candidates as both upstream and downstream effectors of RA signaling. They regulate their target gene expression post-transcriptionally by binding to complementary sequences in the 3' UTRs of target mRNAs, leading to either their degradation or translational repression. Each miRNA can have multiple targets and can regulate complex signaling networks (Bhalala et al., 2013). Various miRNAs have recently been implicated as regulators of epimorphic regeneration of newt lens and hair cells (let-7), newt cardiac muscle (miR-128), fish fins (miR-133a and miR-203), as well as spinal cord regeneration in adult zebrafish (miR-133b) and axolotls (miR-196) (Sehm et al., 2009; Thatcher et al., 2008; Tsonis et al., 2007; Witman et al., 2013; Yin et al., 2008; Yu et al., 2011). However, no miRNAs have as yet been

shown to be involved in the regulation of retinoid signaling in regenerating spinal cords of an adult regeneration-competent species. In the present study, we demonstrate for the first time, a possible role for miR-133a in the regulation of retinoid signaling through the RARβ receptor subtype in the ependymoglial cells of the regenerating adult newt spinal cord.

## 2.03 Materials and Methods

*Animal Care and Surgery*

All procedures were approved by the Brock University Animal Care and Use Committee. Adult eastern spotted newts, *Notophthalmus viridescens*, were obtained from Boreal Science (St. Catharines, Ontario). Animals were housed in plastic containers in dechlorinated water during the experiments, and were fed a diet of liver, brine shrimp or bloodworms three times a week. Prior to all surgical procedures, newts were anaesthetized by soaking in 0.1% tricaine methane sulfonate (MS-222, Sigma) pH 7.0 for 10 minutes. Tails were amputated approximately 1 cm caudal to the cloaca, after which animals were placed on ice for approximately 20 minutes to recover. At each subsequent regenerative timepoint, the above procedure was repeated and blastemas were amputated approximately 1-3 mm rostral to the original site of amputation.

*RT-q-PCR*

Isolated tissues from time 0, 7, 14 or 21 days post amputation (dpa) were immediately flash frozen in liquid nitrogen. Total RNA was extracted as outlined in the Animal Tissue RNA Purification Kit (Norgen Biotek) from uninjured amputated tail at time 0, or from whole blastema at 7, 14 or 21 dpa. Following a quality control test, cDNA was synthesized from 750 ng of RNA according to the SuperScript III Reverse Transcriptase Protocol (Invitrogen), using gene specific stem-loop primers (Varkonyi-Gasic et al., 2007). Quantitative real time PCR (RT-q-PCR) was performed with a 20 µl reaction containing 5 µl of RNase free water, 10 µl of iQ SYBR Green Supermix (BioRad), 1 µl of forward and reverse primers, and 3 µl of cDNA. All reactions had three biological and three technical replicates, and were amplified according to the following

program: 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 64°C for 15 sec and 72°C for 45 sec. The reference genes used for normalization were 5s rRNA and newt Histone Acetyl Transferase. All aspects of the Minimum Information for Publication of Quantitative Real-Time q-PCR Experiments (MIQE) guidelines were adhered to for the above procedures (Taylor et al., 2010), including establishment of equal amplification efficiencies of all reference and target genes. Data were analyzed using the $\Delta\Delta C_T$ method to determine relative expression changes of the target miRNAs during regeneration.

*Locked Nucleic Acid-in situ Hybridization*

Following a modified version of the Nuovo et al. (2009) protocol, tissues isolated from the above timepoints were immediately fixed in neutral buffered formalin, then dehydrated, cleared, and embedded in paraffin. Cross sections 10 μm thick were placed on silane-coated slides, then digested with 200 μl of pepsin for 30 min at room temperature. Locked nucleic acid (LNA) probes labeled with digoxigenin complementary to miR-133a or miR-203 (Exiqon) were diluted with ISH buffer then applied to each section and incubated at 60°C for 5 min then overnight at 37°C. The slides were washed in 0.2X SSC with 2% BSA at 4°C for 5 min, and incubated with 100 μl of antidigoxigenin/alkaline phosphatase for 30 min at 37°C. Following a wash with detection buffer, slides were incubated with NBT/BCIP reagent at 37°C and counterstained with fast red.

*Mimic Injections and Imaging*

miScript miRNA mimics against miR-133a (UUUGGUCCCCUUCAACCAGCUG) and control Scramble (TAACACGTCTATACGCCA) were used for functional analysis (Qiagen).

Oligonucleotides were diluted in water to make a 20 μM stock solution, then further diluted in PBS to make a 160 nM solution for injection. A 10 μl unimetric syringe was used to inject 3 μl of mimic or scramble into the central canal of the spinal cord approximately one hour following amputation. Immediately after injection, electroporation was performed using a Grass SD9 Stimulator (Grass Instruments). Electrodes were placed on dampened filter paper on either side of the tail, and 5 pulses of 25 V, 50 ms each with a 200 ms delay between pulses were given (Sehm et al., 2009). The above procedure was repeated 48 hours post-amputation with injection into the tail regenerate directly caudal to the spinal cord. At each timepoint during the first 21 dpa, regenerating tails were photographed using a dissecting microscope equipped with a Nikon DS-U2 camera and NIS Elements software.

*Western Blot Analysis*

Tissues from animals injected with mimics were flash frozen in liquid $N_2$ immediately after collection, followed by homogenization in lysis buffer and centrifugation. The protein concentration in each sample's supernatant was determined using the BCA protein assay (Pierce). Equivalent amounts of protein from each sample were run at 80 V for 2-3 hr on 12% resolving polyacrylamide gels, and then blotted to nitrocellulose membranes (BioRad). Membranes were blocked in 3% non-fat skim milk powder/0.1% Tween20/PBS for 1 hr, then incubated with primary antibody (1:1000 NvRARβ or 1:10,000 anti-Actin [Ab-Cam] in the above blocking solution) overnight at 4°C. The blots were incubated for 45 min in the dark with Alexa Flour 680 goat anti-rabbit IgG (Invitrogen; 1:15,000 in the above blocking solution), then visualized using an

Odyssey Infrared Imaging System (LI-COR Biosciences). All Western blots included at least three biological replicates and two technical replicates.

*Cloning and Transfection*

RARβ2 3' UTR fragments were amplified from pooled tail RNA (isolated as described above) with the Advantage 2 PCR kit (Clontech) using primers with enzymatic cut sites. The restriction endonuclease used for the forward primer was Nhe1, and for the reverse primer, Sal1. This fragment was then cloned into the pmirGLO Dual Luciferase miRNA Target Expression Vector (Promega). The inserted fragment was confirmed by DNA sequencing. Human PC3 cells were used for transfection of the plasmid alone as well as for co-transfection with the miR-133a mimic or control scrambled oligonucleotide (Qiagen). Transfections were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

*Luciferase Assay*

Human prostatic cancer (PC3) cells were seeded into 96 well white plates (Greiner Cellstar®, Sigma-Aldrich) in DMEM supplemented with high Glucose, non-essential amino acids and 10% Fetal Bovine Serum (all Invitrogen). After reaching ~90% confluence 24 hours later, cells were co-transfected with 100 ng of reporter plasmid and either 20 pmol of miR-133a mimic or scrambled nucleotide, followed by 0.3 µl of Lipofectamine 2000. Forty-eight hours after co-transfection, the luciferase assay was performed using the Dual Glo Luciferase Assay System (Promega). Luminescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer. There were 12 replicates from each experimental group, in which firefly luciferase activity was normalized to the plasmids internal positive control, renilla luciferase activity.

*Statistical Analysis*

The data for the q-PCR assays were analyzed using a One-Way ANOVA with a Post Hoc Tukey test. Western blot data were analyzed using a two-tailed t test. The data for the Luciferase Assay were also analyzed using a One-Way ANOVA with a Post Hoc Tukey test. P-values less than 0.05 were considered statistically significant.

## 2.04 Results

*MicroRNA dysregulation during caudal spinal cord regeneration*

Based on previous data in other model systems which supported roles for miR-133a, miR-124a, miR-132 and miR-203 during epimorphic regeneration or CNS development (Cheng et al., 2009; Thatcher et al., 2008; Vo et al., 2005; Yin et al., 2008), we initially chose to examine the patterns of expression for each of these miRNAs during caudal spinal cord and tail regeneration in the adult newt using quantitative PCR. The expression levels of both miR-133a and miR-132 were significantly decreased compared to levels in unamputated adult tail tissues during the first three weeks following tail amputation (Figs. 2.1A and 2.1B). miR-133a levels were lowest at 14 days post amputation (dpa), declining to roughly 7% of the levels measured in the unamputated tail. miR-133a expression remained low throughout the first 21 dpa.

A significant, roughly 40%, decline in miR-132 transcript levels was first apparent at 14 dpa and was followed by a continual decline to approximately 37% of the levels measured in unamputated tails by 21 dpa (Fig. 2.1B). Conversely, miR-203 expression appeared to gradually increase during the first three weeks post amputation, compared to initial control levels, peaking at more than a 3 fold increase by 21 dpa (Fig. 2.1C). miR-124a is initially increased by more than two fold at 7 dpa before levels return to baseline at 21 dpa. The expression levels of three other variants of the miR-133 family (b, c and d) were examined and found to display an expression pattern similar to that observed for miR-133a (Fig. 2.2). This is not entirely unexpected since the entire miR-133 family can be found within the same highly conserved cluster, which likely has co-regulated expression (Tani et al. 2013). Furthermore, miRNAs within the 133 family

differ by only 1-3 nucleotides, almost exclusively at their 3’ ends and the seed regions of all family members are identical (miRBase.org).

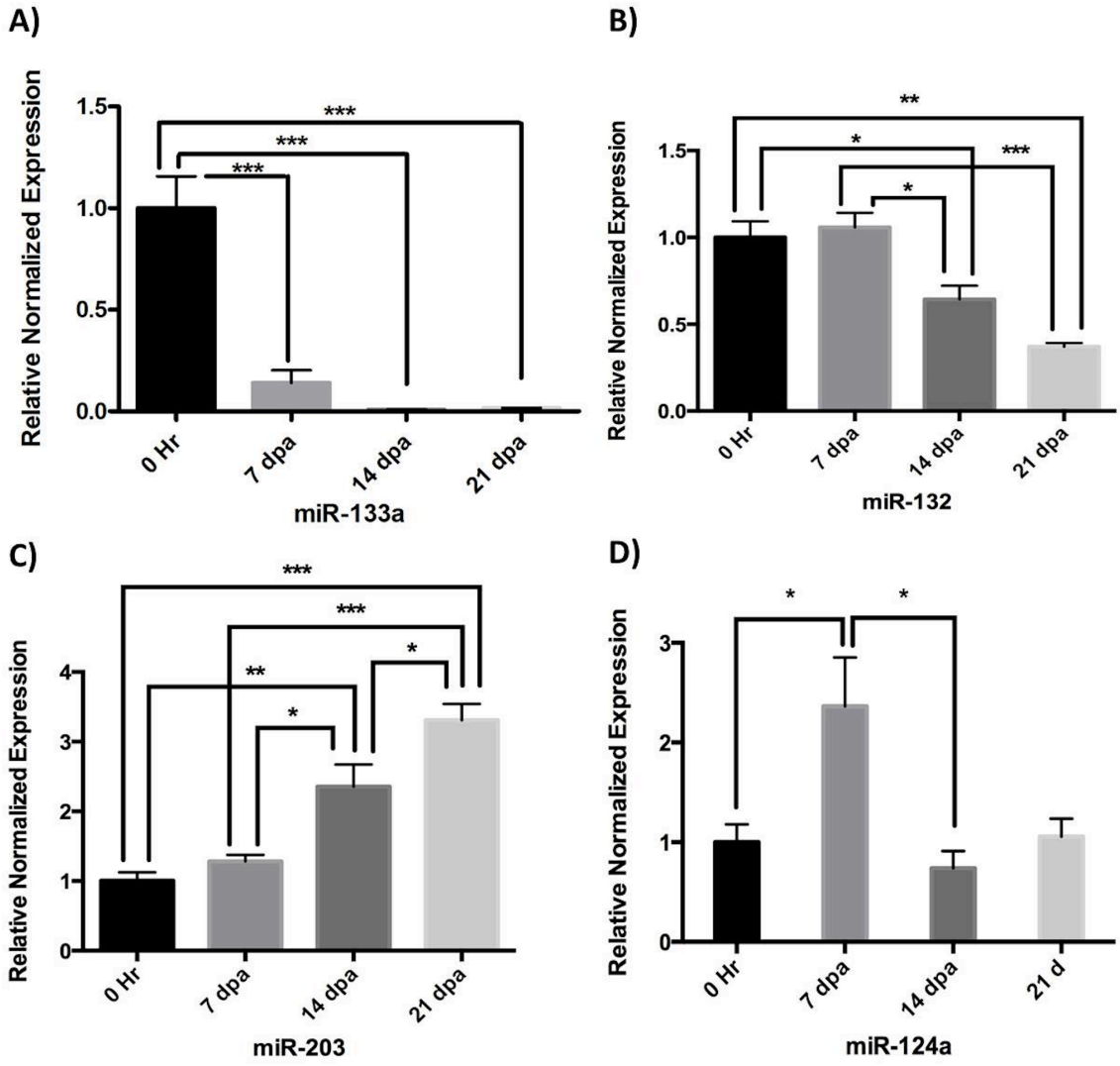


**Figure 2.1.** The expression of miR-133a and miR-132 are down-regulated while miR-203 and miR-124a are up-regulated within the first three weeks after tail amputation. A) miR-133a was significantly down-regulated at 7, 14 and 21 dpa. B) miR-132 was significantly down-regulated at both 14 and 21 dpa. C) miR-203 was significantly increased at 14 and 21 dpa. D) miR-124a was significantly increased at 14 dpa. The normalizing gene used for A) was histone acetyl transferase, for (B-D), 5s rRNA. Error bars indicate standard error. *P<0.05, **P<0.01, ***P<0.001 (n=3).

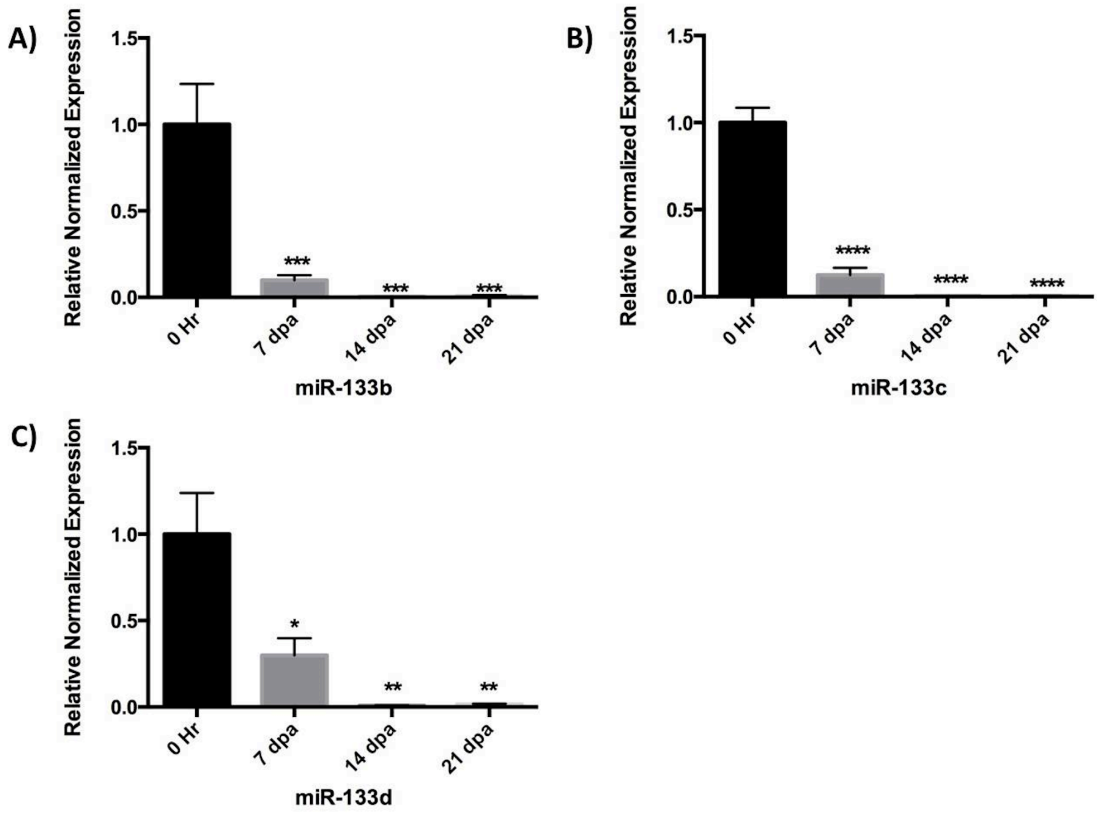


**Figure 2.2.** Each member of the miR-133 family is down-regulated by 7 days post tail amputation. A) miR-133b, B) miR-133c and C) miR-133d were significantly downregulated at 7, 14 and 21 dpa vs. time 0. Error bars indicate standard error. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (n=3).

*miR-133a and miR-203 are expressed primarily in the ependymoglial cells of the spinal cord.*

As mentioned previously, retinoid signaling through RARβ2 is required for functional repair of the newt spinal cord and expression of this receptor is prominent in the ependymoglial cells in the early post amputation stages in tail regenerates (Carter et al., 2011). All three of the miRNAs selected for study have components of the retinoid signaling pathway as putative target mRNAs (see Fig. 2.6A). Thus our next objective was to determine if these microRNAs were expressed in temporal and spatial patterns consistent with their putative role as regulators of retinoid signaling in tail and spinal cord regenerates.

The tissue specific expression pattern of miR-133a and miR-203 were determined using locked-nucleic acid *in situ* hybridization (LNA-ISH). Both miRNAs were expressed primarily in the ependymal cells surrounding the central canal of the spinal cord (Figs. 2.3A, 2.3B, 2.4A). Moreover, the relative levels and time course of expression of both of these miRNAs in the ependymal cells as determined by LNA-ISH, mirror those measured in whole tail/spinal cord regenerate tissue as determined by q-PCR (Figs. 2.3A and 2.3B; compare to Figs. 2.1A and 2.1C). The only other location within the 21 dpa tail regenerates where we were able to detect expression of miR-133a was in mesenchymal precursors for osteocytes and chondrocytes in a regenerating vertebra (Fig. 2.4A). This result is consistent with the recent finding that miR-133a has been shown to regulate mesenchymal cell lineage progression by selectively inhibiting the differentiation of chondrocytes and osteocytes *in vitro* (Zhang et al., 2012).

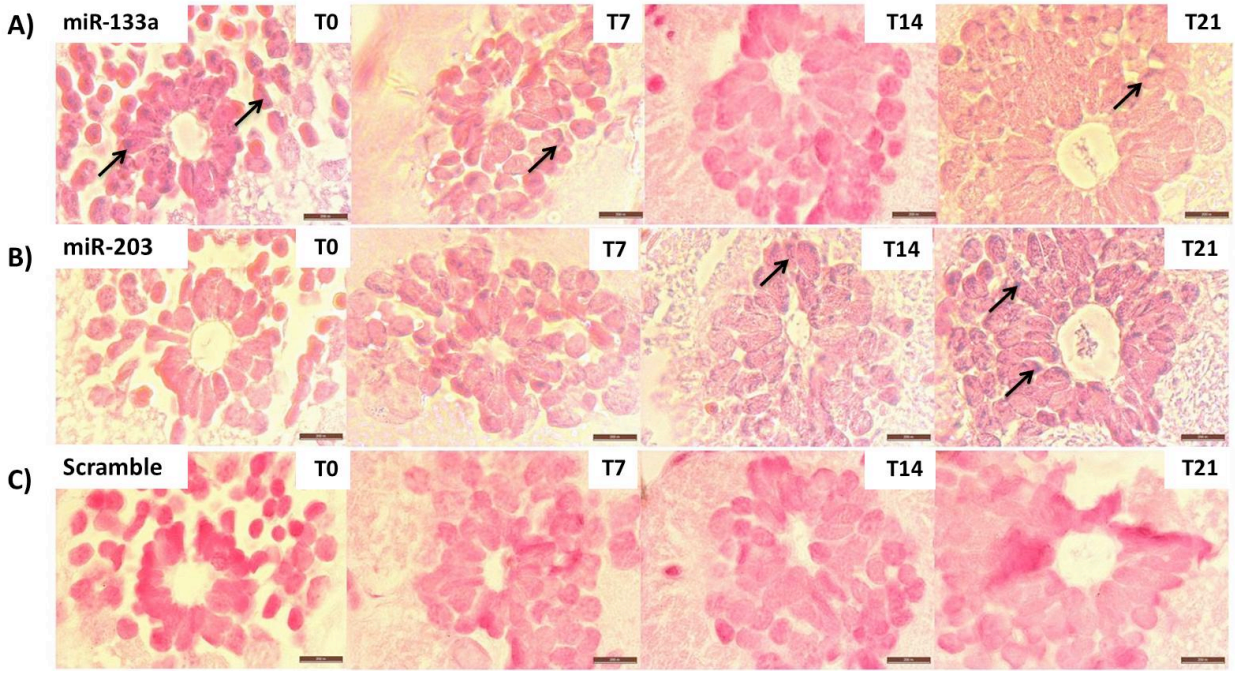


**Figure 2.3.** Both miR-133a and miR-203 are expressed in the ependymal cells surrounding the central canal of the spinal cord. A, B and C represent transverse sections. A) miR-133a expression (blue-purple positive signal) is high in the cytoplasm of ependymal cells rostral to the plane of amputation at time 0, then decreases by day 14 before showing a slight increase at day 21 . B) The expression of miR-203 increases consistently from day 7 to 21 in comparison to unamputated tissues (day 0). C) Sections hybridized with a control scrambled probe show no hybridization signals in any tissues above background. Arrows in all figures represent examples of miR-133a positive cells.

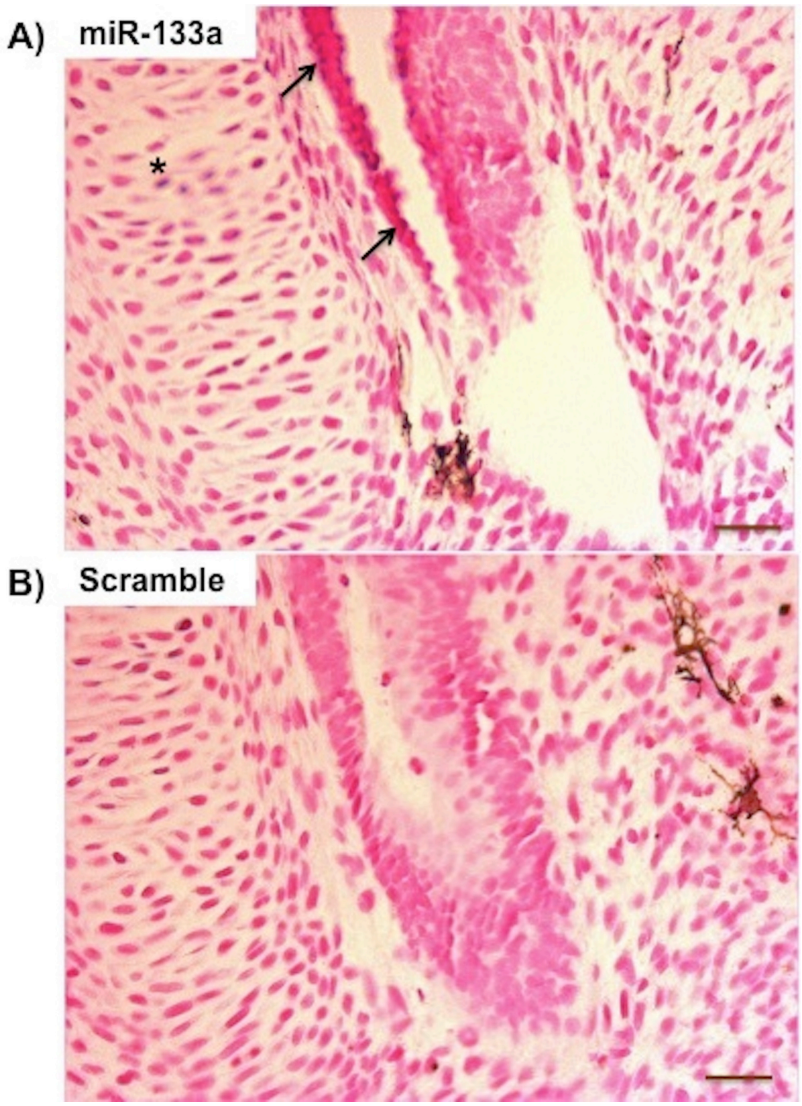


**Figure 2.4.** miR-133a is primarily expressed in the ependymal tube in 21 day regenerates. A and B represent roughly mid-sagittal sections. A) miR-133a expression (arrows) is present within the ependymal tube 21 days after injury. There are also a few miR-133a -positive cells within the mesenchymal precursors for cartilage and bone (asterisks). B) Similar mid-sagittal section hybridized with the negative control scrambled nucleotide probe had no positive signal within either the ependymal tube or any other regenerate tissues.

*miR-133a downregulation in the ependymoglial cells is necessary for spinal cord and tail regeneration.*

To determine if the down-regulation of miR-133a we have detected by qPCR *in vivo* (Fig. 2.1A) is required for spinal cord and tail regeneration, we attempted to alter the level of this miRNA in ependymoglial cells *in vivo* and determine the morphological consequences of such alterations on tail and spinal cord regenerates.

After injection and electroporation *in vivo*, mimic-based up-regulation of miR-133a significantly decreased the rate and extent of tail regeneration compared to a control, scrambled miRNA injection (Figs. 2.5A, 2.5B, 2.5C). Up-regulation of miR-133a by injection and electroporation of the mimic at day 0 pa and again at day 2 pa was confirmed with qPCR at various time points after amputation and injection (Fig. 2.5D). There was a significant increase in miR-133a expression at 7 dpa compared to all other time points. Significantly, early mimic injection at 0 and 2 pa had no significant effect on miR-133a levels at any time point after 7 days. Thus the normal down-regulation of miR-133a appears to be required within the first 7 dpa for normal regeneration of the spinal cord.

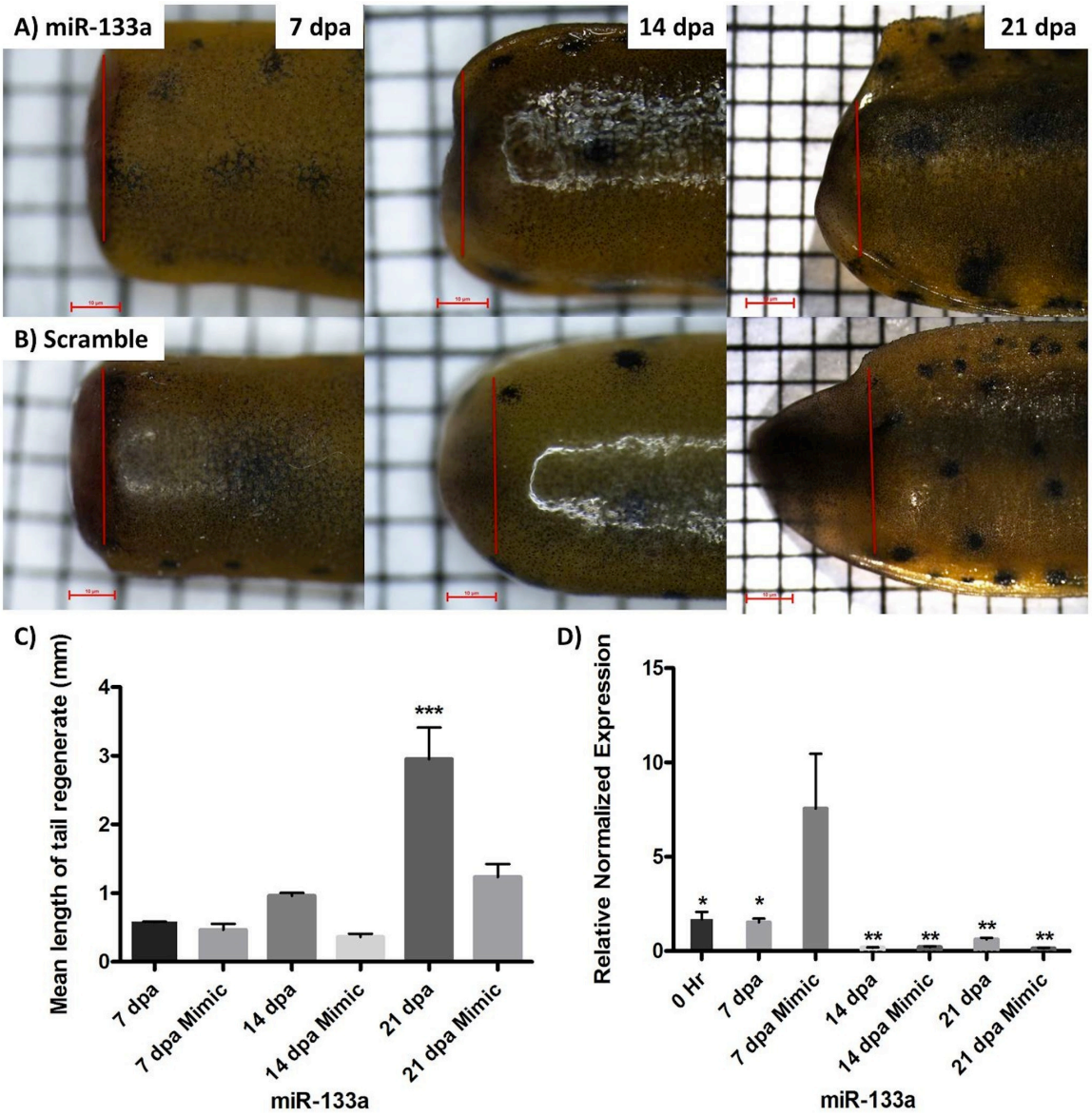


**Figure 2.5.** *In vivo* up-regulation of miR-133a significantly inhibits tail regeneration. A) miR-133a mimic injected tails over the first 21 dpa. Lack of regeneration, (caudal extension and blastema size) is most apparent by 21 dpa. B) Animals injected with a scrambled nucleotide probe exhibit normal regeneration. C) Measurements of regenerate tail length in mimic-133a injected animals show a significant decrease in outgrowth compared to the control-injected animals. D) Confirmation of mimic induced up-regulation of miR-133a by q-PCR. At 7 dpa, miR-133a levels are significantly increased in tails from mimic injected regenerates compared to control, scrambled nucleotide injected regenerates. Error bars indicate standard error. *P<0.05, **P<0.001, ***P<0.001 (n=3).

*miR-133a regulates RARβ2 expression in the ependymoglial cells of the regenerating tail and spinal cord.*

We have previously demonstrated that an upregulation of RARβ2 in ependymoglial cells is a functional requirement for tail and caudal spinal cord regeneration in adult newts (Carter et al., 2011). However, neither the upstream effectors nor the downstream targets of retinoid signaling through this receptor in these tissues have been identified. Since in the present study, we found that miR-133a expression seems to be coincident both temporally and spatially with the expression profile of RARβ2 previously determined by Carter et al. (2011), we initiated a search of the miRanda (microrna.org) database to determine if RARβ2 or any other retinoid signaling pathway components were potential targets for miR-133a (Fig. 2.6A). Potential targets of miR-133a include CRABP 1B and the retinoid receptors RXRα, RARα and importantly, given our previous results, RARβ. A multiple sequence alignment revealed that the seed region of the mature zebrafish dre-miR-133a does have a putative binding site on the previously cloned 3' UTR of the *N. viridescens* RARβ2 (Fig. 2.6B). Thus, we next determined the ability of miR-133a to inhibit expression of a reporter gene containing the 3'-UTR of the newt RARβ transcript. After co-transfection of a miR-133a mimic and the reporter plasmid, pmir-GLO containing the firefly luciferase gene upstream of the newt RARβ 3'-UTR, into PC3 cells, the level of luciferase activity was significantly decreased by greater than 60% (Fig. 2.7). Co-transfection of the same reporter plasmid with a scrambled nucleotide had no significant effect on luciferase activity in the same cell line. PC3 cells do not express any detectable amounts of endogenous miR-133a under similar culture conditions (Tao et al., 2012).

A)

| Target | MicroRNA |
|---|---|
| RDH10 | miR-203 |
| CYP26 | miR-124a |
| CRABP1B | miR-133a |
| RXRα | miR-133a |
| RARα | miR-133a-d |
| RARβ | miR-124a, miR-133a |

B)

```
RARβ2      GAAGTTAATTGACTTTTTAAGACATTCATATTAAAAAAAAACCTTATACACAGCTAGGAA
miR-133a         TCGACCATTT                                  TAC
                 * ***  ***                                  ***

RARβ2      AACCCCTCATGGCTTGGTTTCCAGAACAAAAAAAAAAAAAAAAAAAAA
miR-133a         CTTGGTTTA
                 ********
```

**Figure 2.6.** A) Predicted targets of miRNAs 124a, 133a-d and 203 include components of the retinoid signaling pathway. B) Sequence alignment of the 3' UTR of the N. viridescens RARβ2 mRNA with mature dre-miR-133a displays potential binding sites. Notably, the first complement includes the entire seed region of miR-133a.

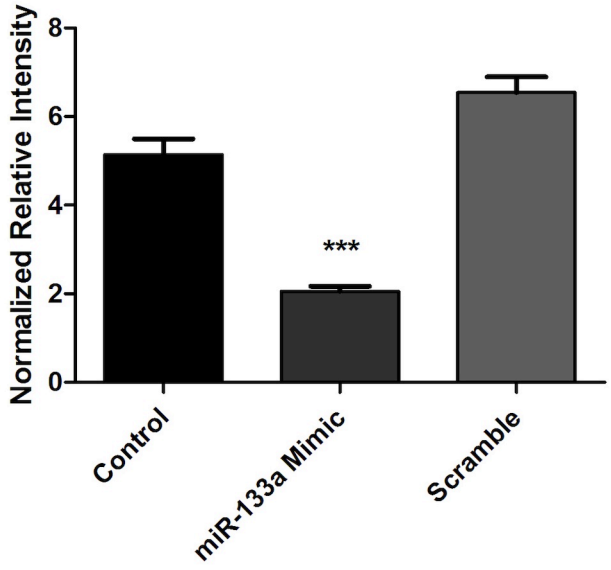


**Figure 2.7.** Newt RARβ mRNA is a direct target of miR133a *in vitro*. The pmiR-Glo luciferase reporter assay verified direct binding of miR-133a with putative binding sites on the 3'-UTR of newt RARβ mRNA. Control = Relative luciferase activity from the pmiR-Glo plasmid in PC3 cells containing the newt RARβ 3'-UTR in the absence of exogenous miR-133a. The addition of the miR133a mimic to the cells led to a 60% decline in luciferase activity while the addition of a scrambled nucleotide had no significant effect. N= 12 in all cases. *** $p < .001$.

Given these results *in vitro*, our next aim was to determine whether newt RARβ2 is a target of miR-133a *in vivo*. To do so, we compared the levels of RARβ2 protein, as determined by Western blotting, after injection and electroporation of a miR-133a mimic, to those measured after similar treatment with the control scrambled miRNA (Fig. 2.8A). RARβ2 protein levels were found to be lower in those tissues where miR-133a was upregulated at both 7 and 14 dpa, with the decrease at day 14 significant compared to control injected regenerates (Fig. 2.8B, $p<0.001$). The average level of RARβ protein at day 7 after mimic injection was also lower than that for the regenerates receiving the scrambled nucleic acid, however the p value was greater than 0.05 ($n=3$; $P= 0.186$). Thus together, our *in vitro* and *in vivo* data support the hypothesis that RARβ transcripts represent targets for miR-133a regulation within the ependymal cells of the regenerating caudal spinal cord in adult newt tails.

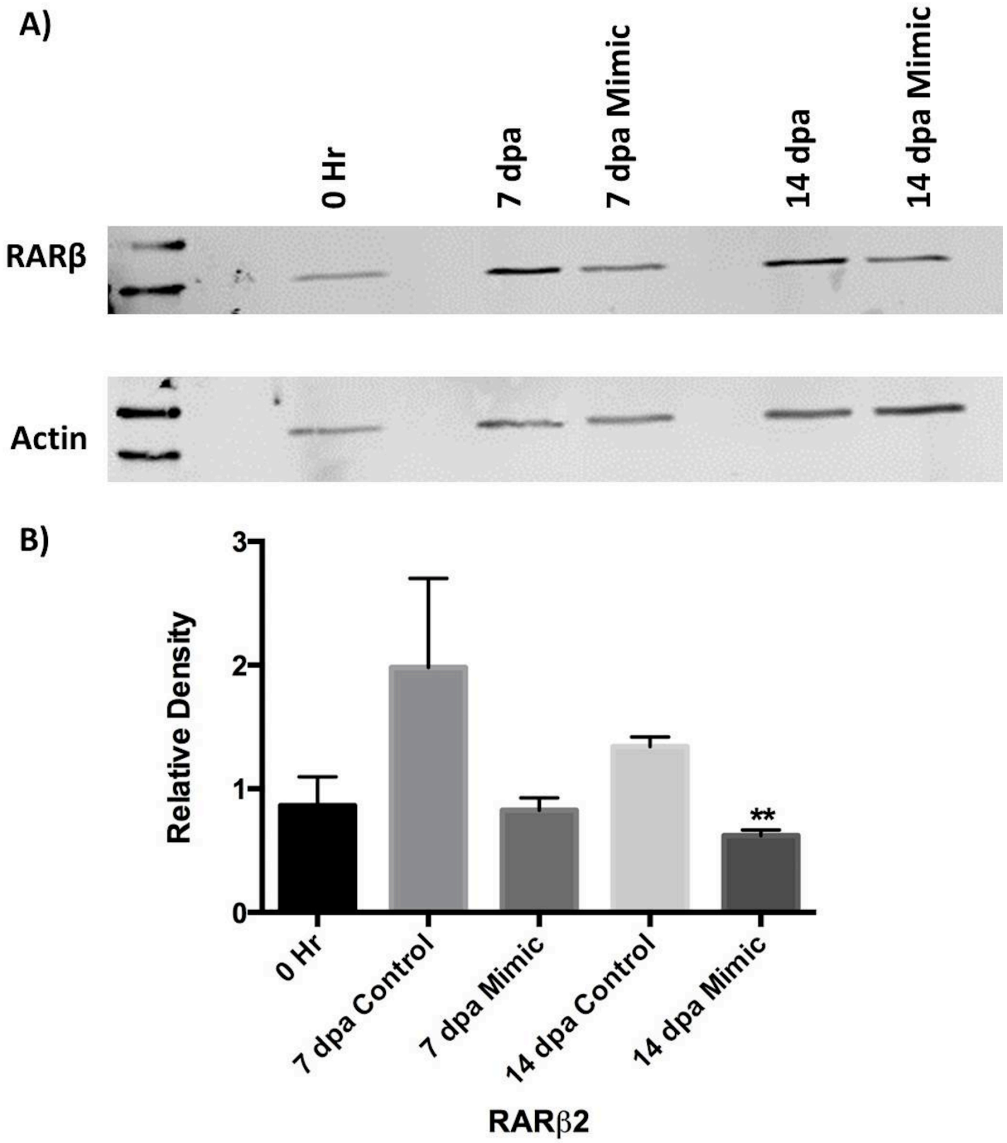


**Figure 2.8.** miR-133a targets RARβ in the newt tail during regeneration. A) Expression of RARβ was down-regulated at 14 dpa in response to mimic-based up-regulation of miR-133a. B) Histogram depicting changes in RARβ expression shown in (A). Levels of RARβ2 were significantly decreased 14 dpa in the mimic vs. control. The relative density of RARβ2 was determined by normalizing the RARβ2 signal density to the signal density of the control protein, actin. Error bars represent standard error. An unpaired t test was used to compare RARβ levels in control regenerates 14 dpa to 14 dpa miR-133a mimic-injected regenerates. **P<0.001 (n=3).

**2.05 Discussion**

Due to their ability to target multiple mRNAs and thus regulate complex gene expression networks simultaneously, miRNAs are potentially critical regulators in the recovery from and repair of spinal cord injuries in regeneration-competent species such as urodele amphibians and zebrafish. Indeed, a number of groups have previously examined miRNA expression patterns during lens and tail regeneration in urodeles (Sehm et al., 2009; Tsonis et al., 2007), as well as during fin and spinal cord regeneration in zebrafish (Thatcher et al., 2008; Yin et al., 2008; Yu et al., 2011).

In the present study, we initially examined the levels of four microRNAs during the course of tail and caudal spinal cord regeneration in the adult newt. Two of these, miR-133a (as well as other members of the miR-133 family) and miR-132, were significantly downregulated during the first 21 days post amputation of the tail. Two others were either continually (miR-203) or transiently (miR-124a) up-regulated during the course of tail regeneration. Our results with Q-PCR, in some cases are consistent with and in others, are inconsistent with published microarray analyses of miRNA expression in epimorphic regeneration of limbs (fins) or tail and spinal cord in regeneration-competent species (Sehm et al., 2009; Yin et al., 2008; Yu et al., 2011). For example, we see a consistent but transient increase in miR-124a expression at 7 days after tail amputation in the adult newt, whereas Sehm et al. (2009) show a consistent decrease (confirmed by Q-PCR) at 3 days post amputation of the tail in the axolotl. It is possible that miR-124a is also downregulated in the newt tail at an earlier stage of regeneration and upregulated at a later stage in axolotl tail regeneration. In addition, our results were obtained in adult newts whereas their analyses were performed on relatively young

axolotlss of between 2-3 cm. Differences in expression of miR-124a between these two urodele species would not be surprising, since the cellular mechanisms underlying regeneration have been shown to differ between these two urodele species in other tissues (Sandoval-Guzmán et al., 2014). In addition, previous studies on spinal cord regeneration support the concept that different cellular mechanisms may underlie SCI repair in these two urodele species (Chernoff et al., 2003; Zukor et al., 2011).

miR-124a expression is abundant in the vertebrate CNS and increases during development, reaching maximal levels in mature neurons (Cao et al., 2007; Cheng et al., 2009; Lagos-Quintana et al., 2002). Yu et al. (2008) have shown that overexpression of miR-124a in mouse PC19 cells and primary cortical neurons promotes neurite outgrowth by targeting mRNAs for proteins that mediate cytoskeletal dynamics. Others have shown that overexpression of miR-124a promotes the differentiation of neural stem cells and may play a role in the repair of spinal cords in a rat model of SCI (Xu et al., 2012). Curiously, the levels of miR-124a were found to decrease significantly between days 1 and 7 after a compression trauma to the rat spinal cord. miR-124a was not detected by in situ hybridization around the injury site at day 7 post injury. Xu et al. (2012) did not determine the levels of miR-124a at any time points after 7 days. Perhaps the transient increase we observe in the levels of miR-124a at 7 days post amputation in the newt reflect an important difference between a regeneration competent and regeneration incompetent species with respect to the promotion of ependymal cell proliferation or neurite outgrowth in response to spinal cord injury in these two species. Indeed, the newt spinal cord regenerative response at day 7 includes extensive ependymal cell proliferation

and extension of an ependymal tube and terminal bulb beyond the original amputation plane (Iten and Bryant, 1976).

In our work, the expression of miR-133a significantly decreased in the ependymoglial cells of the newt caudal spinal cord compared to the expression levels in undamaged tail tissues. This downregulation of miR-133a is similar to that seen in regenerating zebrafish fins (Yin et al., 2008) and in regenerating spinal cord tissue in the axolotl by microarray analysis (Sehm et al., 2009). In the zebrafish fin, miR-133a levels are initially high in unamputated fin tissue but are significantly reduced as early as 2 days after fin resection. This reduction in miR-133a was shown to be dependent upon an increase in fibroblast growth factor (FGF) signaling post-amputation as part of the regeneration program leading to blastema cell proliferation and redifferentiation of fin tissues. Yin et al. (2008) thus suggested that miR-133a acts as a "regenerative brake" within a complex regulatory circuit that must be "released" by FGF receptor activation. As a consequence, one predicted target of miR-133a, the monopolar spindle 1 (Mps1) kinase, an established regulator of zebrafish blastema cell proliferation (Poss et al., 2002), is active at the appropriate time and in the appropriate cells to promote blastema cell accumulation and subsequent redifferentiation.

More recently, Yu et al. (2011) have demonstrated that another miR-133 family member, miR-133b, is essential for functional recovery after spinal cord injury in adult zebrafish. miR-133b had earlier been shown to enhance muscle regeneration and reduce fibrotic tissue in a rat skeletal muscle injury model (Nakasa et al., 2010). Unlike our present results with miR-133a, however, Yu et al. (2011) found a significant increase in miR-133b expression, albeit in cell bodies of regenerating neurons within the nucleus

medial longitudinal fasciculus (NMLF) in the brainstem, as soon as 6 hours and as long as 7 days after spinal cord transection. This same increase was not seen at the injury site. One predicted target of miR-133b is the ras homolog A (RhoA) (Chiba et al., 2009), whose downregulation had been shown to enhance regrowth of the corticspinal tract and decrease tissue damage and cavity formation in a rat spinal cord injury model (Fournier et al., 2003). Yu et al. (2011) provided evidence that altering the levels of miR-133b with an antisense morpholino oligonucleotide at 9 days after SC injury increased RhoA levels in the brain and spinal cord. We did not examine the effects of a downregulation of miR-133a on RhoA levels at the injury site or in any tissues of the regenerate tail in the newt. It would be interesting to determine whether the decrease in miR-133a that we see after 7 days post amputation in the newt tail is matched by a concomitant increase in RhoA levels at that stage.

We have, however, demonstrated for the first time, indirectly, that miR-133a may target RARβ2 mRNA in ependymoglial cells *in vivo.* A multiple sequence alignment revealed that the seed region of the dre-miR-133a has at least one and possibly three binding sites within the 3' UTR of the cloned newt RARβ2. Indeed, results from our *in vitro* luciferase reporter assay confirm that the 3' UTR of the newt RARβ2 gene represents a target for miR-133a since only a miR-133a mimic and not a scrambled nucleotide of equivalent length inhibited luciferase activity. When the same miR-133a mimic was injected and electroporated into the ependymal layer of the amputated cord at the site of injury at two time points within the first 48 hours after amputation, the levels of RARβ2 protein significantly decreased compared to those injected with scrambled nucleotides. Moreover, both the decrease in miR-133a levels seen in this study and the

increase in RARβ2 protein we have seen in our previous work are, for the most part, spatially restricted to the ependymoglial cells rostral to the amputation plane. This is significant in that these cells may be multipotent neural stem cells that divide and migrate caudally as a consequence of injury to the tail in the axolotl, ultimately giving rise to both glial cells and neurons of the regenerated cord (Benraiss et al., 1996; Echeverri and Tanaka, 2002; Mchedlishvili et al., 2012). In support of this concept, at least one factor associated with pluripotency in stem cells, FGF2, is increased in radial glia cells in adult newt spinal cords after injury (Zhang et al., 2000). This may again represent the trigger necessary to unleash the "regenerative brake" established by miR-133a in the undamaged cord (Yin et al., 2008), leading to miR-133a down-regulation. As a consequence, RARβ2 protein levels would increase in the ependymal cells as previously demonstrated by Carter et al. (2011). It is still unclear what genes are regulated downstream of RARβ2 in the ependymoglia within the first week post amputation of the newt tail. Some of these genes may include other miRNAs (Gudas, 2013). Indeed we have preliminary data identifying numerous miRNAs that are dysregulated in regenerating newt spinal cord and tail tissues as a consequence of inhibiting either retinoid signaling with a selective antagonist of RARβ2 or retinoic acid synthesis with the aldehyde dehydrogenase inhibitor, DEAB. Studies aimed at elucidating the spatial and temporal patterns of expression of some of these miRNAs, as well as their putative *in vivo* mRNA targets are underway. These will hopefully lead to a clearer picture of the complex signaling pathways underlying epimorphic regeneration in this species and highlight the role of retinoid signaling and miRNAs in neural stem cell activation and differentiation in these tissues.

**2. 06 Acknowledgements**

The authors would like to thank Gaynor Spencer for critical comments on the manuscript, Rachel Nottrodt for assistance with statistical analyses of the data, David Rozema for assistance with the LNA *in situs*, and Jeff Stuart, Chris Carter and João Fonseca for assistance with the cloning, PC3 cell culture and luciferase assays.

## CHAPTER THREE:

## MicroRNA dysregulation in response to RARβ2 inhibition reveals a negative feedback loop between microRNAs 1, 133a and RARβ2 during tail and spinal cord regeneration in the adult newt.

**Submitted:** to Developmental Dynamics on April 27th, 2015.

**3.01 Abstract**

The molecular events and pathways underlying epimorphic regeneration of the adult urodele amphibian tail and caudal spinal cord are not well understood. Two regulatory pathways that may intersect to regulate these complex events are microRNAs (miRNA) and retinoic acid (RA) signaling. Recent evidence supports a role for miR-133a targeting a specific retinoic acid receptor subtype (RARβ2) within the ependymoglial cells of the regenerating spinal cord in adult newts. Given the dynamic nature of gene expression control by RA and the pleiotropic effects of miRNAs on multiple mRNA targets in this complex system, we chose to examine whether RA signaling through RARβ2 alters miRNA expression in tissues of the regenerating tail and spinal cord. A miRNA microarray screen identified 18 highly conserved miRNAs that were differentially expressed in regenerating tail and spinal cord tissues after inhibition of RARβ2 signaling with a selective antagonist, LE135. Using qPCR, the expression patterns of several of these miRNAs were found to be dysregulated in response to RARβ2 inhibition over the first 21 days of tail regeneration. Interestingly, miRNAs let-7c, miR-1 and miR-223 were expressed within the ependymoglial cells surrounding the central canal of the spinal cord, coincident with the previously identified expression domain of RARβ2. Altering the endogenous expression pattern of these three miRNAs, by injection and *in vivo* electroporation of specific mimics or inhibitors, led to a significant inhibition of regeneration by 21 days post amputation (dpa). Furthermore, we have shown that miR-1 targets the 3' UTR of RARβ2 mRNA *in vitro*; and *in vivo,* mimic-based upregulation of miR-1 led to a significant decrease in RARβ2 protein. These and previous data suggest that miR-1 and miR-133a, both members of the same

miRNA gene cluster, may participate with RARβ2 in a negative feedback loop and contribute to the regulation of the ependymal response after tail amputation.

### 3.02 Introduction

Injury to the mammalian spinal cord often results in permanent deficits such as paralysis, and even premature death. The primary injury elicits immediate tissue damage, followed by the secondary injury, which consists of inflammation, glial scarring and white matter inhibitors (Abu-Rub et al., 2010). Together, these factors form a chemical and physical barrier that is inhibitory for axonal regrowth, and damaged axons are incapable of regenerating beyond the site of injury to promote functional recovery. Some species of urodele amphibians, such as the Eastern Red-Spotted newt, *Notophthalmus viridescens*, possess the remarkable ability to regenerate and recover function after a spinal cord injury (Butler and Ward, 1967, 1965; Chernoff et al., 2003; Diaz Quiroz and Echeverri, 2013; Iten and Bryant, 1976; Mchedlishvili et al., 2012; Zukor et al., 2011). The precise mechanisms that allow for spinal cord regeneration are poorly understood, and the identification of factors involved in creating an environment permissive for regrowth are active areas of research.

One such factor is all-trans retinoic acid (RA), a biologically active metabolite of vitamin A, which is vital during the patterning, development, and regeneration of the central nervous system (CNS) (Blum and Begemann, 2013; Duester, 2008; Maden, 2007; Monaghan and Maden, 2012). Traditionally, RA enters its target cell's nucleus and binds to ligand activated nuclear transcription factors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), inducing a change in gene expression. There are at least three subtypes of RARs and RXRs ($\alpha$,$\beta$, and $\gamma$), each with multiple isoforms (Maden et al., 1998). It is abundant throughout the developing CNS, particularly within the spinal cord (Duester, 2008; Maden, 2007). It may also act as a chemotactic factor in the

developing CNS, as it has been shown to stimulate directed neurite outgrowth in embryonic mouse dorsal root ganglia (DRGs), specifically through the RARβ receptor subtype (Corcoran et al., 2000). This receptor subtype may also mediate neurite outgrowth *in vitro* and some functional regeneration after spinal cord injury in rats postnatally (Agudo et al., 2010; Wong et al., 2006; Ping K Yip et al., 2006).

In urodele amphibians capable of regenerating lost structures, RA has been shown to play a role in proximal-distal repatterning in regenerating limbs (Pecorino et al., 1996), as well as enhancing the proliferation and differentiation of the wound epithelium and blastema mesenchyme (Maden et al., 1998; Viviano and Brockes, 1996). In the newt, exogenous RA has been shown to act as a chemoattractant for adult spinal cord neurites *in vitro,* an effect also mediated by RARβ (Dmetrichuk et al., 2005). Expression of RARβ in the adult newt is localized to the ependymoglial cells surrounding the central canal of the spinal cord prior to injury, and is upregulated within these cells by 7 days post tail amputation (Carter et al., 2011). These ependymoglial cells represent an important progenitor source for both neural and non-neuronal cells during the regeneration process in urodele spinal cord and brain (Echeverri and Tanaka, 2002; Kirkham et al., 2014; Maden, 2013). The fact that RARβ expression is enhanced in these neural stem cells following tail and caudal spinal cord regeneration support a putative role for this nuclear receptor in the control of neural progenitor cell proliferation and neurite guidance during caudal tail regeneration.

Very little research to date has focused on the factors that act upstream or downstream of RARβ-mediated retinoid signaling during the ependymal response in caudal spinal cord regeneration. Recently, microRNAs (miRNAs) have been implicated

in neural regeneration in this and other species (Diaz Quiroz et al., 2014; Sehm et al., 2009). miRNAs represent a class of small, non-coding RNAs approximately 22 nucleotides in length. They act post-transcriptionally to regulate gene expression by binding to the 3' UTR of their target mRNA (Bhalala et al., 2013). Each miRNA can have multiple target mRNAs, allowing them to regulate complex signaling pathways, such as those that are active during regeneration, when global changes in gene expression occur rapidly in response to injury. For example, miRNAs have been implicated during the regeneration of the newt lens (let-7), newt cardiac muscle (miR-128), zebrafish fin (miR-133a and miR-203), zebrafish spinal cord (miR-133b) as well as axolotl spinal cord (miR-125b and miR-196) (Diaz Quiroz et al., 2014; Sehm et al., 2009; Thatcher et al., 2008; Tsonis et al., 2007; Witman et al., 2013; Yin and Poss, 2008; Yu et al., 2011). Most recently, we have demonstrated that miR-133a is expressed in the ependymoglial cells of the uninjured spinal cord of the adult newt, and that it is significantly downregulated in response to caudal tail amputation (Lepp and Carlone, 2014). Furthermore, we provided evidence that miR-133a targets the 3' UTR of newt RARβ2 in ependymoglial cells, and that experimental upregulation of miR-133a *in vivo* delays tail regeneration by inhibiting RARβ2 expression (similar to treatment with LE135- a RARβ-selective antagonist (Carter et al., 2011)). In the present study, our main objective was to determine, by microarray analysis, which miRNAs may act as downstream effectors of RARβ2-mediated retinoid signaling in the regenerating newt tail and spinal cord. We found 18 miRNAs that were significantly up- or downregulated in response to RARβ2 inhibition. Importantly, we provide evidence *in vivo* for a role for three of these (miR-1, miR-223 and let-7) miRNAs in the ependymoglial cells during the early stages of tail and spinal

cord regeneration. Additional evidence is provided in support of a negative feedback loop that may involve both miR-1 and miR-133a, resulting in the maintenance of RARβ2 expression within the ependymoglia during caudal spinal cord regeneration.

### 3.03 Materials and Methods

*Animal Handling and Surgery*

All procedures were approved by the Brock University Animal Care and Use Committee. Adult eastern spotted newts, *Notopthalmus viridescens,* were purchased from Boreal Science (St. Catharines, Ontario). Newts were housed in plastic containers with dechlorinated water on a 12 hr. light-dark cycle and were fed liver, brine shrimp and blood worms three times a week. Newts were anaesthetized by immersion in 0.1% tricaine methane sulfonate (MS-222, Sigma), pH 7.0, for 10 minutes prior to any surgical procedures. Amputation of tails was performed approximately 1 cm caudal to the cloaca, which was followed by 20 minutes on ice for recovery. When each regenerative timepoint was reached, newts were again anaesthetized, and blastemas were collected by cutting the regenerate 1-3 mm rostral to the original plane of amputation.

*LE135 Treatment*

Once animals regained consciousness post tail amputation, they were immediately placed in a dechlorinated water bath containing either $10^{-6}$ M LE135 in DMSO, an RARβ selective antagonist (a kind gift from H. Kagechika, Tokyo), or 0.01% DMSO, the vehicle control (Carter et al., 2011). The solution bath was changed twice weekly, and animals were maintained in these treatments for up to 21 days. Tail regenerates were then surgically removed at various timepoints as described above for microarray analysis and qPCR. We have previously shown that regardless of whether LE135 is applied via bath application or by implantation of AG1-X2 beads into the central canal of the cut end of the spinal cord, tail regeneration is inhibited to the same degree and RARβ2 expression is downregulated in the regenerate tissues by 21 days pa (Carter et al., 2011).

*Microarray Analysis*

Two days post amputation, blastemas were isolated from animals within each treatment group (DMSO or LE135) and immediately flash frozen in liquid nitrogen. Following homogenization, total RNA was isolated from the tissues at various timepoints using the Animal Tissue RNA Purification Kit (Norgen Biotek, St. Catharines, Ontario). RNA quality was confirmed via spectrophotometry and gel electrophoresis, and three blastemas from each treatment were pooled for testing. The microarray analysis was outsourced to LC Sciences (Houston, Texas). The platform used for miRNA microarrays by LC Sciences is the μParaflo®Microfluidic Biochip, with *in situ* synthesized probes. Total RNA (2 μg) from each treatment group was hybridized to its own custom Cy3 chip to allow for statistical comparison between the DMSO and LE135 treated blastema RNA. Each chip contained probes complementary to the known miRNAs of *Danio rerio*, *Xenopus laevis* and *Xenopus tropicalis*, amounting to 265 miRNAs in total, which did not include the endogenous chip controls (Sanger miRBase Release 16.0). Each detection probe on each chip was tested in triplicate. Regression based mapping was used to subtract background from the array data, which was then normalized using a locally-weighted scatter plot smooth (LOWESS) filter. Clustering and statistical analysis were also performed by LC Sciences, and only signals that were differentially expressed at a significant level ($p<0.01$) were considered for further study.

*miRNA Quantification (RT-qPCR)*

Tissue was isolated at one of four timepoints, 0, 7, 14 or 21 days post tail amputation (dpa) and immediately flash frozen in liquid nitrogen. These tissues were

homogenized and RNA was extracted as described above. Using 750 ng of total RNA, cDNA was synthesized using gene specific stem-loop primers (Varkonyi-Gasic et al., 2007), in accordance with the SuperScript III Reverse Transcriptase kit (Invitrogen). Quantitative real time PCR (qPCR) was run using iQ SYBR Green Supermix (BioRad) on a CFX Connect Real-Time System (BioRad). A 20 μl reaction was used which contained the following; 5 μl of RNase free water, 10 μl of iQ SYBR Green Supermix, 1 μl of forward and reverse primers and 3 μl of cDNA. The following program was used for amplification; 95° for 5 min, followed by 40 cycles of 95° for 15 sec, annealing temp for 15 sec (64° for miR-1, miR-26a and miR-133a; 66° for miR-145 and let-7c; 68° for miR-223 and miR-1306). All reactions were run with three biological and three technical replicates, and were normalized to two reference genes (5s rRNA and β-actin), and analysed using the $\Delta\Delta C_T$ method. The Minimum Information for Publication of Quantitative Real-Time qPCR Experiments (MIQE) were adhered to for the above experiments (Taylor et al., 2010). The primers used for gene specific cDNA synthesis as well as qPCR are listed in Table 1:

**Table 3.1.** Primer sequences for cDNA synthesis and qPCR.

| |
|---|
| RT-miR-145: |
| GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGATT |
| RT-miR-223: |
| GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGGTAT |
| RT-miR-1: |
| GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATACATA |
| RT-let-7c: |
| GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACCATA |
| RT-miR-26a: |
| GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCCTAT |
| RT-miR-1306: |
| GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACCA |
| RT-miR-133a: |
| GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCTGG |

Forward-miR-145: GCCGCGTCCAGTTTTCCCAGG
Forward-miR-223: GCCGCTGTCAGTTTGTCAAATACC
Forward-miR-1: GCCGCTGGAATGTAAAGAAG
Forward-let-7c: GCCGCTGAGGTAGTAGGTTG
Forward-miR-26a: GCCGCTTCAAGTAATCCAGG
Forward-miR-1306: GCCGCTTGGCTCTGGTGG
Forward-miR-133a: GCCGCTTTGGTCCCCTTCA
Reverse: GTGCAGGGTCCGAGGT

*Locked Nucleic Acid in situ Hybridization*

Uninjured tail tissue, as well as blastemas from 7, 14 and 21 dpa were isolated and immediately fixed in neutral buffered formalin. Following ethanol dehydration, clearing and infiltration, tissues were embedded in paraffin, 10 μm sections were obtained and floated onto silane-coated slides. A modified version of the methods of Nuovo et al. (2009) was followed for *in situ* hybridization using locked nucleic acid (LNA) probes tagged with digoxigenin complementary to let-7c, miR-1 or miR-223 (Exiqon). Sections were first digested with 200 μl of pepsin for 30 min at room temperature, followed by a 10 min wash with 0.1 M triethanolamine (TEA), a 10 min wash with TEA and acetic anhydride, and 4 washes with PBS. Slides were incubated with 125 pmol of probe for 5 min at 60°C, and hybridized overnight at 50°C. Following a wash in 0.2X SSC with 2% BSA at 4°C for 5 min, sections were incubated with 100 μl of antidigoxigenin/alkaline phosphatase (Roche) at 37°C for 30 min. After a brief wash in detection buffer, slides were held at 37°C with NBT/BCIP (Enzo Life Sciences) while being monitored for colorimetric change prior to counterstaining with Fast Red (Enzo Life Sciences) and fixation with Permount.

*Inhibitor and Mimic Injections*

A scrambled sequence oligonucleotide control (TAACACGTCTATACGCCA), LNA inhibitor against miR-223 (TGGGGTATTTGACAAACTGA) (Exiqon), or

miScript mimics against miR-1 (UGGAAUGUAAAGAAGUAUGUAU) and let-7c (UGAGGUAGUAGGUUGUAUGGUU) (Qiagen) were used for *in vivo* functional analyses. RNase free water was added to each oligonucleotide to make a 20 μM stock solution, which was then further diluted in PBS to 200 nM. In order to allow for any bleeding to subside, injections did not take place until at least 1 hour after amputation. A 10 μl unimetric syringe was used to inject 3 μl of scramble, mimic or inhibitor into the central canal of the spinal cord. *In vivo* electroporation was performed immediately following injection. Briefly, dampened filter paper was placed on either side of the tail underneath flat circular electrodes, then 5 pulses of 25 V, 50 ms each with a 200 ms delay was applied using a Grass SD9 Stimulator (Grass Instruments). Injections and electroporations were repeated at 2 dpa and 6 dpa into the blastema, directly distal to the ependymal bulb. Prior to isolating tissue at each timepoint, regenerates were photographed using a dissecting microscope equipped with a Nikon DS-U2 camera and NIS Elements software. Alteration of total miRNA levels after the application of mimics or inhibitors was confirmed using qPCR for each timepoint.

*Western Blotting*

Tissue from animals injected with the miR-1 mimic were collected at 7, 14 and 21 dpa and immediately flash frozen. Following homogenization in lysis buffer and centrifugation for 30 min at 4°C, the total protein concentration of each sample's supernatant was determined using the BCA protein assay (Pierce). 20 μg of protein from each sample were run for 2 hours at 80 V on 12% mini-PROTEAN precast acrylamide gels (BioRad). Following blotting to nitrocellulose (BioRad), membranes were blocked in 3% non-fat skim milk powder/0.1% Tween20/PBS for 1 hr. Membranes were then

incubated with primary antibody, 1:1000 *Nv*RARβ2 (custom antibody, Pacific Immunology), or 1:500 anti-α-Tubulin (Developmental Studies Hybridoma Bank, The University of Iowa), in blocking solution for 12 hours at 4°C. Blots were then incubated with 1:15000 secondary antibody, Alexa Flour 680 goat anti-rabbit IgG (Invitrogen) (for RARβ2) or Alexa Flour 680 goat anti-mouse IgG (Invitrogen) (for Tubulin) for 45 min. An Odyssey Infrared Imaging System (LI-COR Biosciences) was used to obtain blot images. The analysis was comprised of three biological and two technical replicates.

*Cloning, Transfection and Luciferase Assay*

The 3' UTR of RARβ2 was previously amplified and cloned into the pmirGLO Dual Luciferase miRNA Target Expression Vector (Promega), using the restriction endonucleases *Nhe1* and *Sal1* (Lepp and Carlone, 2014). The cells used for transfection were from a Human PC3 cell line, seeded into 96 well plates (Greiner Cellstar®, Sigma-Aldrich), using DMEM supplemented with non-essential amino acids, high glucose and 10% Fetal Bovine Serum (Invitrogen). The transfection agent used was Lipofectamine 2000 according to the manufacturers protocol. 100 ng of the plasmid was transfected into each well, once the cells reached ~90% confluence. Samples were co-transfected with either 20 pmol of miR-1 mimic (Qiagen) or scrambled oligonucleotide (Exiqon). After 48 hrs, Firefly and Renilla luciferase activities were determined using the Dual Glo Luciferase Assay System (Promega) with a Varian Cary Eclipse fluorescence spectrophotometer. Each of the three experimental groups (plasmid alone, plasmid & mimic, plasmid & scramble) was replicated 12 times, and firefly luciferase activity was normalized to the internal *Renilla* luciferase activity for analysis.

*Statistical Analysis*

A two-tailed student's t-test was used to compare DMSO and LE135 relative expression for the miRNA probe repeats on each custom microarray chip. Data for all qPCR studies were analyzed using a One-Way ANOVA with a Post Hoc Tukey test. The length of regenerates and Western Blot data from the injections studies were analyzed using a two-tailed t-test to compare mimic/inhibitor with matched controls. The firefly luciferase activity was also analyzed using a One-Way ANOVA with a Post Hoc Tukey test. $P<0.05$ was considered statistically significant for most studies; for the microarray analysis, $P<0.01$ was considered significant.

## 3.04 Results

*Microarray expression profiling of miRNAs as putative targets of retinoid signaling*

Just as miRNAs can regulate complex patterns of gene expression post-transcriptionally, their expression may in turn be controlled transcriptionally by the activities of various signaling pathways. Thus, to determine which, if any, miRNAs were putative effectors (either indirectly or directly) of retinoid signaling through RARβ2, a miRNA microarray analysis was conducted. Pooled RNA samples were isolated from tail regenerates 48 hours after transection and treatment with either LE135, an RARβ-selective antagonist, or the vehicle control (a 1:1 (v:v) mixture of DMSO and EtOH). It has previously been demonstrated that treatment with LE135 inhibited tail and caudal spinal cord regeneration in the newt and significantly downregulated RARβ2 protein levels by 21 dpa (Carter et al., 2011). miRNA profiling was outsourced through LC Sciences, using a probe set that included 265 miRNAs from *Danio rerio*, *Xenopus laevis* and *Xenopus tropicalis*.

Eighteen miRNAs were significantly ($p < 0.01$) differentially expressed when comparisons between treatment groups were analyzed after background subtraction and normalization (Figs. 3.1A, 3.1B). Interestingly, miR-133a displayed a significant upregulation after LE135 treatment, suggesting that RARβ2 signaling may negatively regulate the expression of this miRNA in the regenerating tail. This was somewhat surprising since we had previously demonstrated that miR-133a acts upstream of, and directly targets RARβ2 in the ependymal cells of the tail during spinal cord regeneration (Lepp and Carlone, 2014).

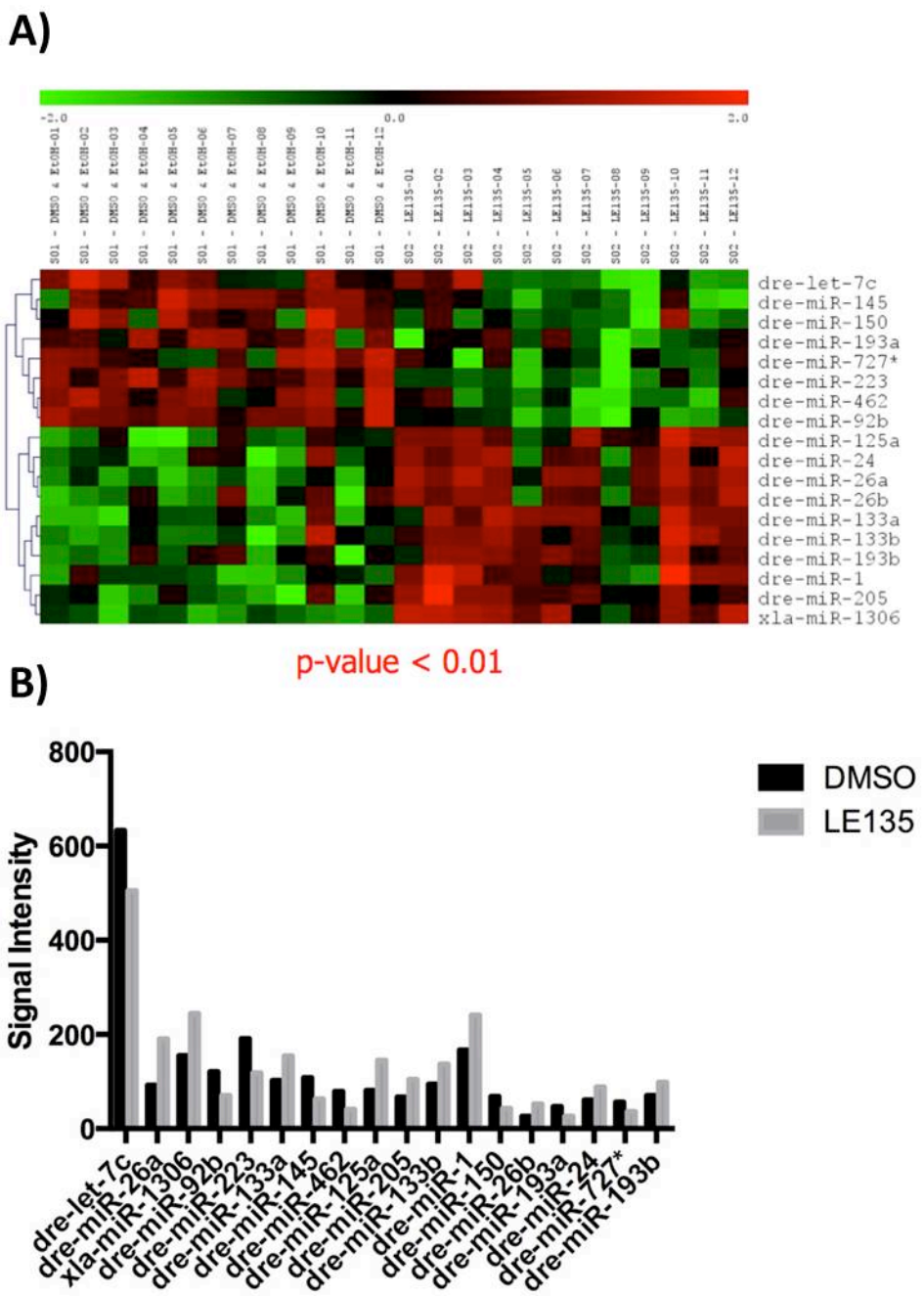


**Figure 3.1.** miRNA expression is dysregulated by inhibition of RARβ signaling during tail regeneration in newts. A) Microarray heat map expression profile depicting relative expression levels of the 18 miRNAs that were significantly different between DMSO (control) and LE135-treated 2 dpa regenerates (P<0.01). Rows represent miRNAs of interest and columns represent tests run, where a green signal indicates downregulation while a red is indicative of an upregulation. B) The signal intensities for each treatment group for the 18 miRNAs that were significantly differentially expressed in the microarray screen. Each chip was run in triplicate.

Among other miRNAs identified by this analysis, of particular interest were miR-1, miR-26a, miR-145, miR-223, miR-1306 and let-7c. Each of these miRNAs has been implicated previously in the regulation of apoptosis, inflammation and stem cell maintenance or proliferation, all cellular functions associated with epimorphic regeneration (Kyritsis et al., 2012; Liu et al., 2009; Melton et al., 2010; Morin et al., 2008; Nakanishi et al., 2010; Petrie et al., 2014).

*MicroRNA expression is dysregulated in response to inhibition of RARβ2 signaling.*

Next, using RT-qPCR, expression profiles for each of the seven miRNAs listed above were established at 7, 14 and 21 dpa for tail regenerates from both LE135 and DMSO treated animals (Fig. 3.2). With the exception of let-7c, miR-1306, miR-223 and miR-145, the expression profiles of each of the remaining 4 miRNAs (miR-1, miR-26a, and miR-133a) were consistent with the microarray data obtained from RNA isolated at 2 dpa, displaying the same pattern of dysregulation in expression levels between treatment groups (Figs. 3.2A-D, 3.2F). The levels of let-7c and miR-223 ($p<0.05$), as well as miR-26a and miR-1 ($p<0.01$), were significantly higher in regenerates treated with LE135 at 14 dpa compared to those treated with DMSO (Fig. 3.2A-D). The levels of expression for both miR-1306 and miR-145 were not significantly different between treatment groups at any time-point examined (Fig. 3.2E, 3.2G). miR-133a levels are significantly higher (compared to DMSO treatment) in regenerates from animals treated with LE135 at 21 dpa ($p<0.01$) but not at 14 dpa, although a similar (but not statistically significant, $p< 0.0539$) trend is apparent at day 14 as well (Fig. 3.2F).

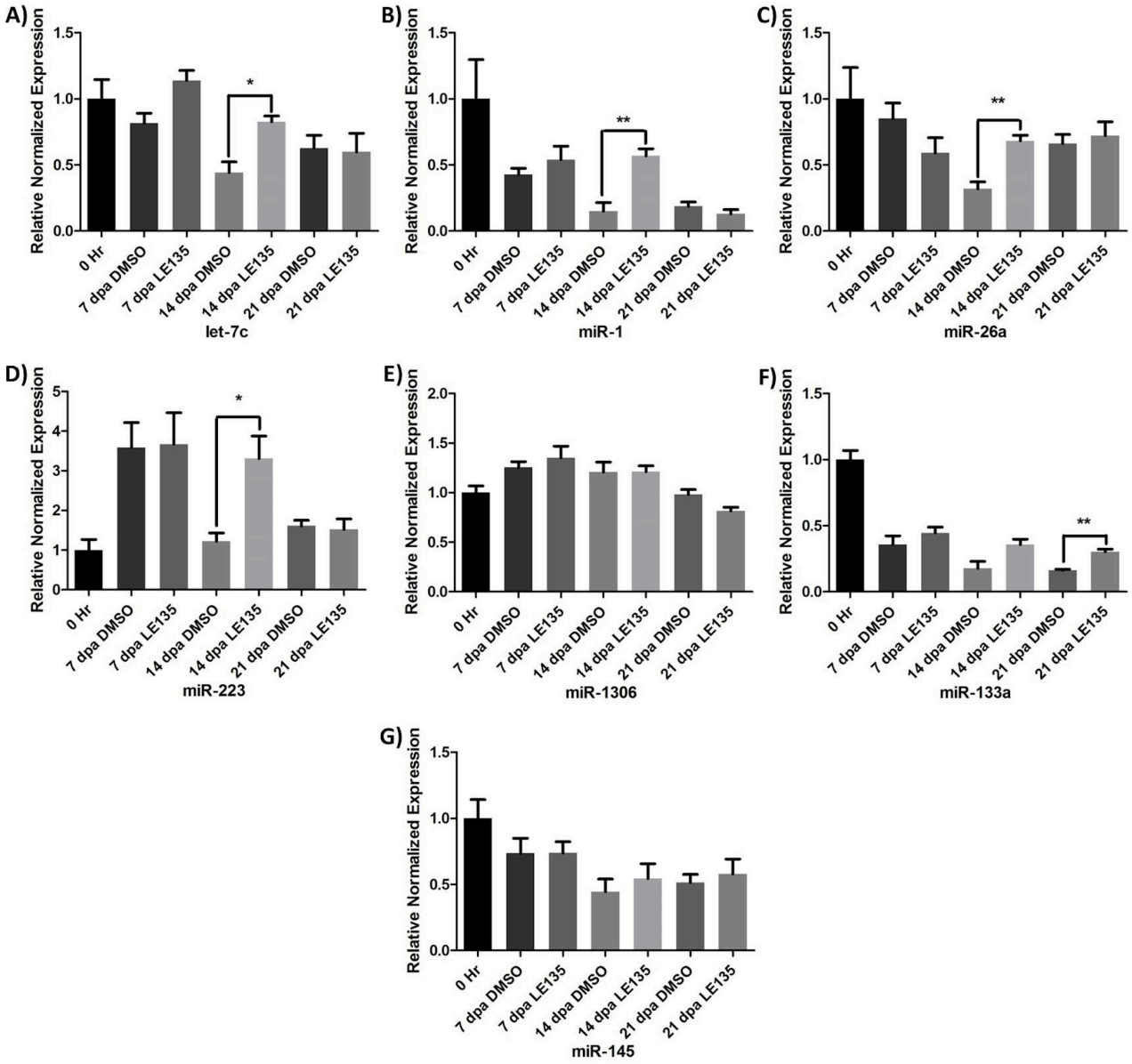


**Figure 3.2.** Expression profile for seven miRNAs at various regeneration time points. Regenerates were obtained from animals treated with DMSO or $10^{-6}$ M LE135. Graphical representation of qPCR analyses for A) let-7c, B) miR-1, C) miR-26a, D) miR-223, E) miR-1306, F) miR-133a and G) miR-145 at 7, 14 and 21 dpa normalized to time 0. Error bars indicate standard error. * $P<0.05$, ** $P<0.01$ (n=3).

These results are intriguing as Carter et al., (2011) had previously demonstrated that RARβ2 expression is significantly upregulated by 14 dpa, particularly within the ependymal layer of the caudal spinal cord, in the regenerating tail in this species. Thus, by experimentally inhibiting retinoid signaling via RARβ2 with LE135, the levels of miRNAs let-7c, miR-1, miR-26a, miR-223 and miR-133a are maintained at abnormally high levels at 14 or 21 dpa. The regeneration stage-specific increase in RARβ2 by day 14 in tail regenerates (Carter et al., 2011) may thus contribute, under normal circumstances, to either a sustained (miR-1, miR-133a and miR-223) or transient (let-7c and miR-26a) decrease in the expression of these miRNAs after 14 dpa (Figs. 3.2 A-D, F). This could, in turn, contribute to an increase in the abundance of their specific target mRNAs.

*let-7c, miR-1 and miR-223 are expressed within the ependymal cell layer of the spinal cord.*

Our data demonstrate that the normal (after DMSO treatment) patterns of expression of some of these miRNAs change temporally, either coincident with or subsequent to, the upregulation of RARβ2 (Fig. 3.2) (Carter et al., 2011). Thus, our next objective was to determine whether the spatial patterns of expression of these miRNAs were consistent with their putative role as targets of RARβ2-mediated retinoid signaling. Since we had previously established that miR-133a is expressed in the ependymoglial cells of the regenerating spinal cord (Lepp and Carlone, 2014), we chose to concentrate in this study on let-7c, miR-1 and miR-223. Indeed, both miR-1 and miR-223 have been reported in other systems to be involved in the regulation of inflammation (Bhalala et al., 2013; Izumi et al., 2011; Nakanishi et al., 2010; Strickland et al., 2011), while let-7c is involved in the regulation of stem cell renewal (Melton et al., 2010). Not only are these

processes necessary for epimorphic tail regeneration in the newt, but retinoid signaling has also been implicated in both inflammation and stem cell maintenance in other systems (Choschzick et al., 2014; Galli et al., 2013; Sarang et al., 2014). In order to characterize their spatial pattern of expression, digoxygenin labeled LNA probes against let-7c, miR-1 and miR-223 were used for *in situ* hybridization on tissue sections of normal, uninjured (untreated with either DMSO or LE135) or regenerating tails at 7, 14 and 21 dpa. As can be seen in Figs. 3.3, 3.4 and 3.5, expression of each of these miRNAs is apparent in the ependymoglial cells immediately surrounding the central canal of the spinal cord in those tissues within an area 1 mm rostral to the original amputation plane. It would appear that expression is localized to the nuclei of these cells, however the nuclei of ependymal cells are very large, and it is difficult to discern whether expression is in fact nuclear or cytoplasmic. However, recent evidence suggests that miRNAs localized to the nucleus can still function in a similar manner as they would in the cytoplasm (Roberts, 2014). Regardless of whether these miRNAs are within the cytoplasm or nuclei, they are associated, almost exclusively, with ependymoglial cells. These same cells had previously been shown to contain the highest levels of RARβ2 at both 7 and 14 dpa by Carter et al., (2011).

A scrambled oligonucleotide was used as a negative control, and only background staining was evident within the ependymal cells at any time points examined (Fig. 3.6). let-7c expression is present within the ependymal cells in uninjured tails and appears to increase by 7 dpa (Figs. 3.3A, 3.3B), followed by a decrease to almost background levels by 14 and 21 dpa (Figs. 3.3C, 3.3D). Similarly, miR-1 expression is high within the majority of ependymal cells in the intact tail and spinal cord, but then drops off in these

same cell types by 7 dpa and is undetectable by days 14 and 21 dpa (Fig. 3.4). Expression of miR-223 on the other hand, is virtually undetectable in the ependymoglial layer at the time of injury, but increases by 7 dpa

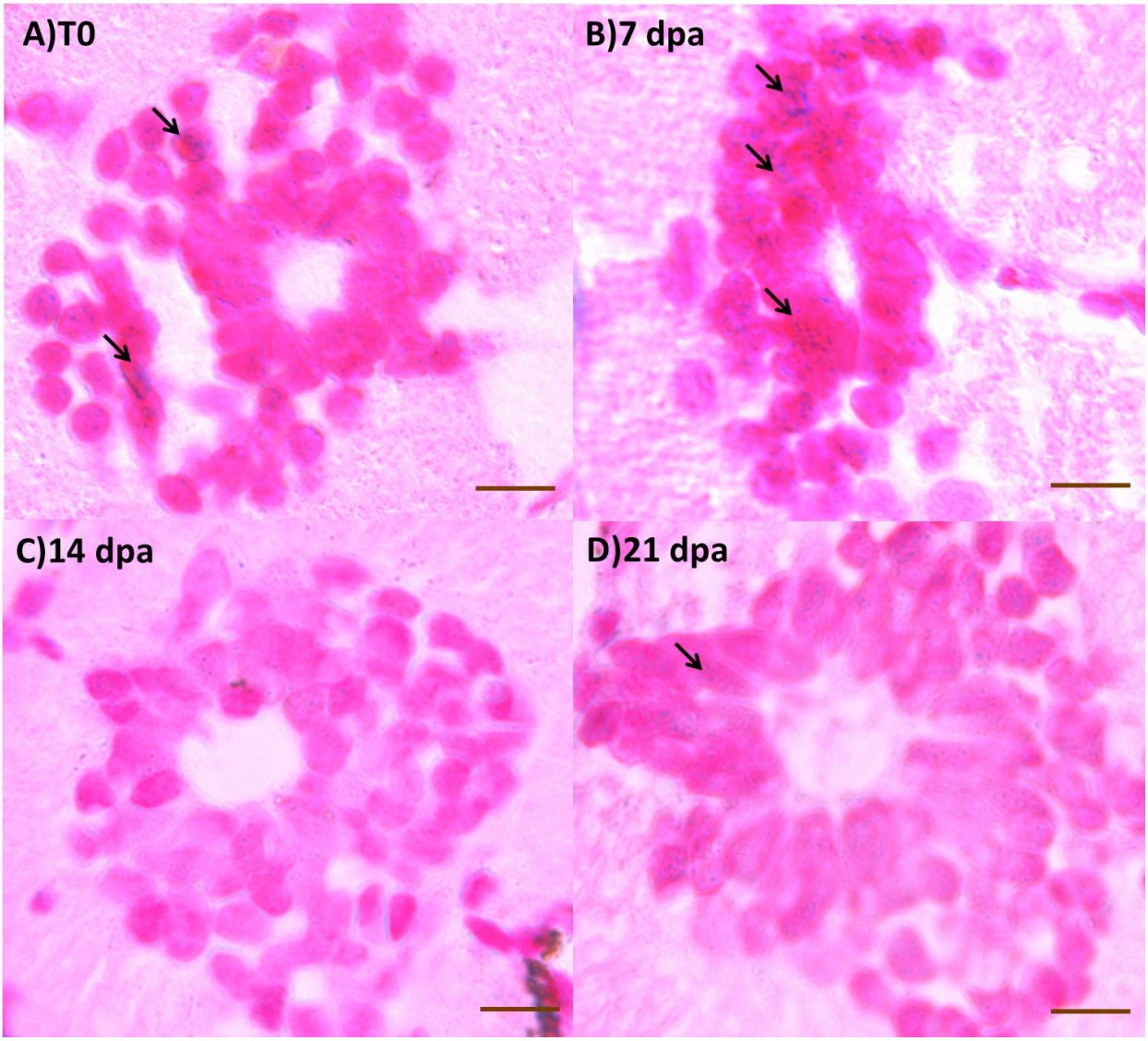


**Figure 3.3.** Expression of let-7c is downregulated by 14 dpa and confined to the ependymal cells surrounding the central canal of the spinal cord. A blue-purple positive signal is indicated by arrows on cross-sections of tail tissues at time 0 (A), 7 dpa (B), 14 dpa (C) and 21 dpa (D). Scale = 200 μm

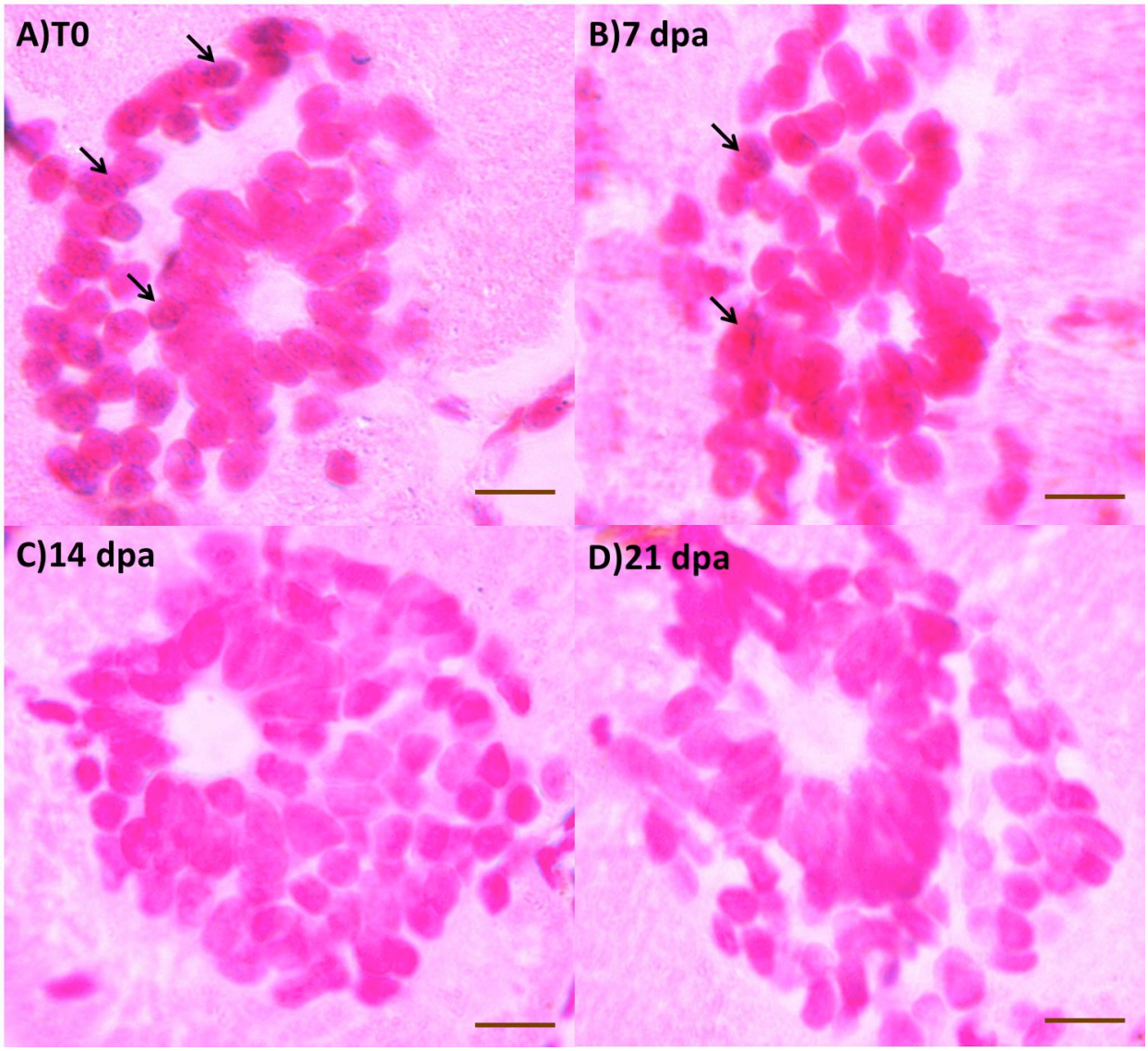


**Figure 3.4.** miR-1 expression is downregulated in ependymal cells after amputation of the tail. (A) Expression is abundant in the ependymal cells at the time of amputation (arrows indicate positive blue signal), but downregulated at 7 and 14 dpa (B, C). By 21 dpa, expression has started to increase again (D), but does not appear to reach the levels seen at day 0. Cross sections, Scale bar = 200 μm.

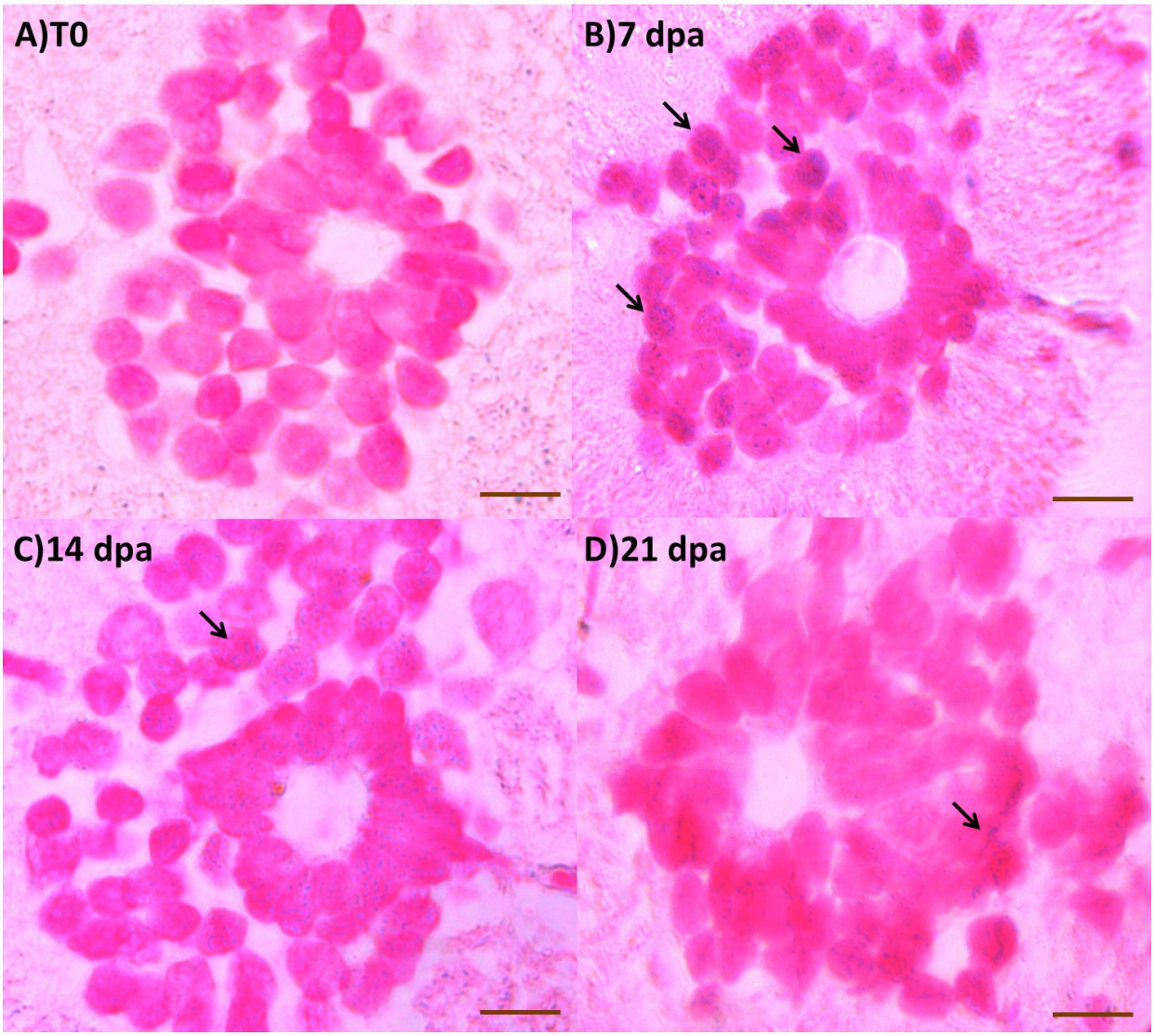


**Figure 3.5.** Expression of miR-223 is upregulated in the ependymal cells after tail amputation. (A) Expression of miR-223 is low at 0 dpa, but upregulated by 7 dpa within these ependymal cells (B). There is still some expression (although less abundant than at 7 dpa) evident at 14 and 21 days within these cells (C, D). Cross-sections, Scale bar = 200 μm.

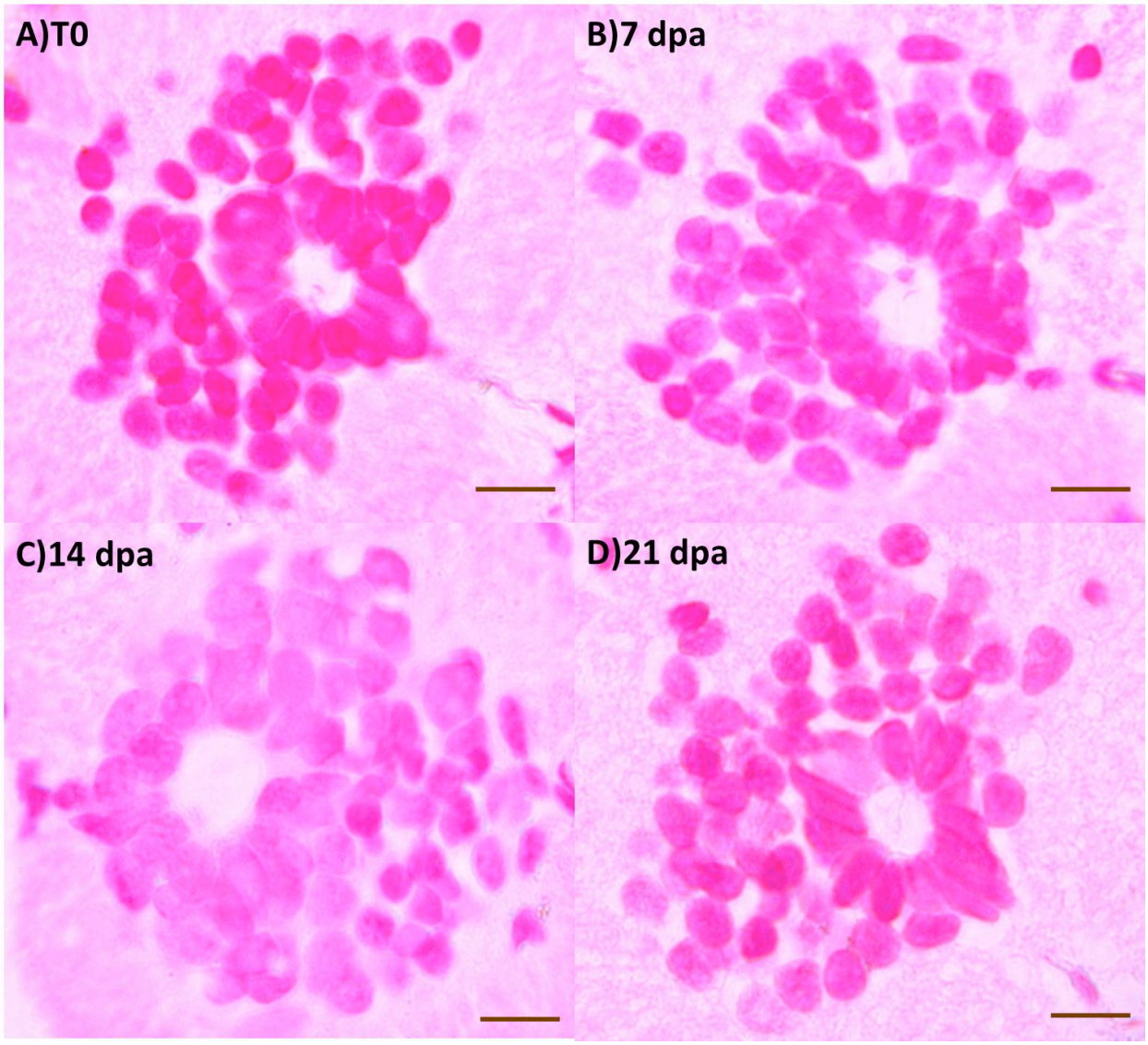


**Figure 3.6.** Tissues hybridized with a control, scrambled oligonucleotide probe show only background signal on cross sections taken from regenerates at 0 dpa (A), 7 dpa (B), 14 dpa (C) and 21 dpa (D). Cross-sections, Scale bar = 200 μm.

(Fig. 3.5A, 3.5B), and gradually diminishes to background levels by 21 dpa in these cells (Figs. 3.5C, 3.5D).

The relative stage-specific pattern of expression of each of these miRNAs as determined by LNA *in situ* hybridization in normal regenerates are consistent with the patterns determined by q-PCR from regenerates treated with DMSO (Compare Fig. 3.2 to Fig. 3.3, 3.4, 3.5). Furthermore, each of these miRNA levels are diminished beyond 14 dpa, at a stage when the expression of RARβ2 is highest within ependymal tissues of the regenerating tail and caudal spinal cord. Taken together, these data provide support for miR-1, let-7c and miR-223 functioning downstream of and being negatively regulated by RARβ2-mediated retinoid signaling.

*The stage-specific downregulation of miR-1 and let-7c and the upregulation of miR-223 within the first 14 dpa are required for spinal cord and tail regeneration.*

To determine if the normal downregulation of miR-1 and let-7, or upregulation of miR-223 that we have detected in ependymoglial cells *in vivo* are required for tail and spinal cord regeneration, we attempted to experimentally alter the concentrations of these miRNAs *in vivo.* Injections into the central canal of the spinal cord followed by *in vivo* electroporation of either synthetic oligonucleotide mimics for miR-1 or let-7c, or an inhibitor of mature miR-223, were carried out immediately after tail amputation and were repeated at both 2 dpa and 6 dpa beneath the wound epithelium, immediately distal to the regenerating ependymal bulb.

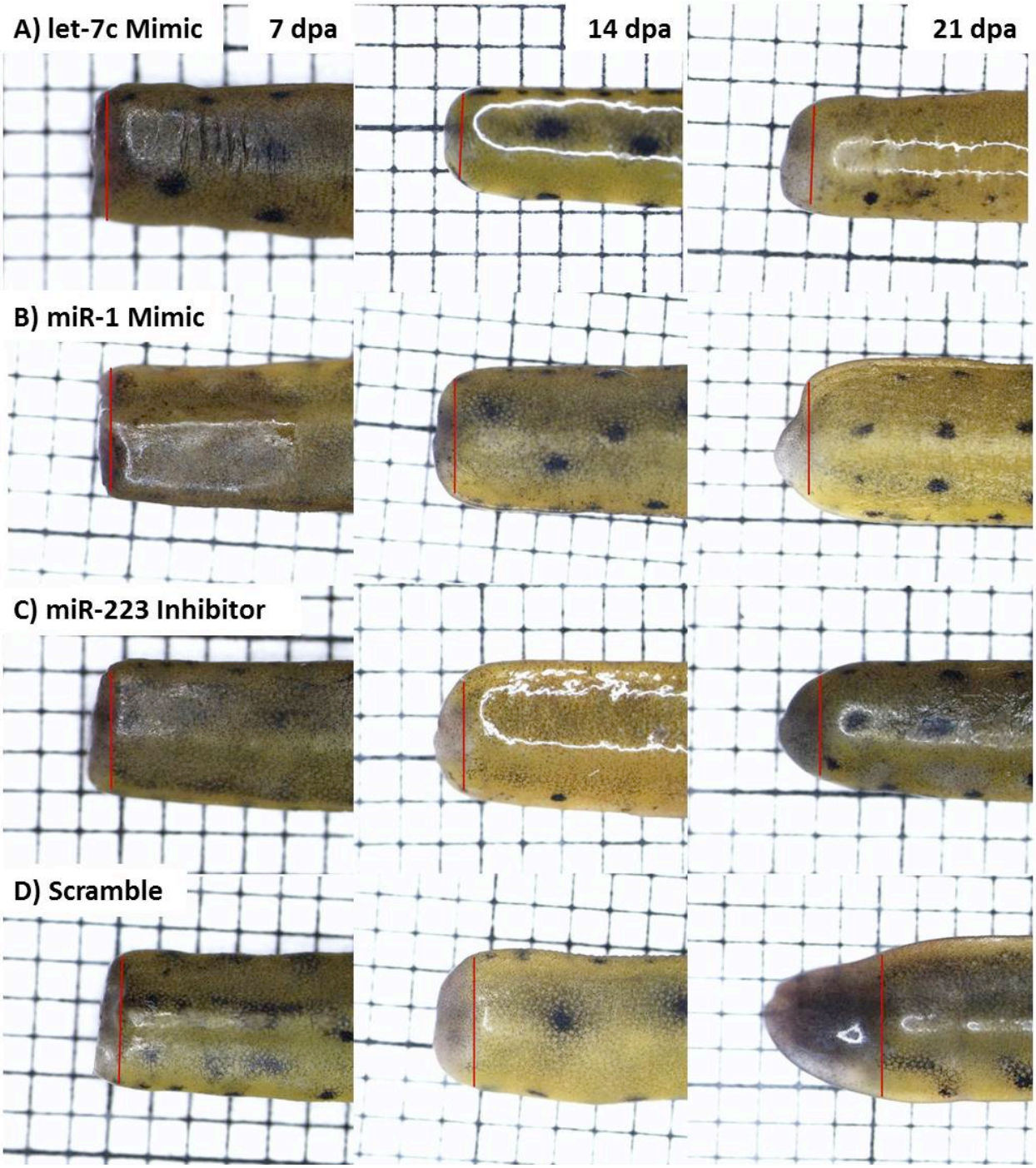


**Figure 3.7.** In vivo injection of let-7c and miR-1 mimics or miR-223 inhibitors significantly inhibits tail regeneration. (A-D) Micrographs showing the extent of tail regeneration over the first 21 dpa after in vivo injection and electroporation of (A) a let-7c mimic, (B) a miR-1 mimic or (C) a miR-223 inhibitor. (D) Tail regeneration was unaffected by the injection of a scrambled oligonucleotide. In all cases, regenerate lengths were shorter in animals injected with mimics or inhibitor compared to those injected with the scrambled oligonucleotide. Scale bar = 1 mm.

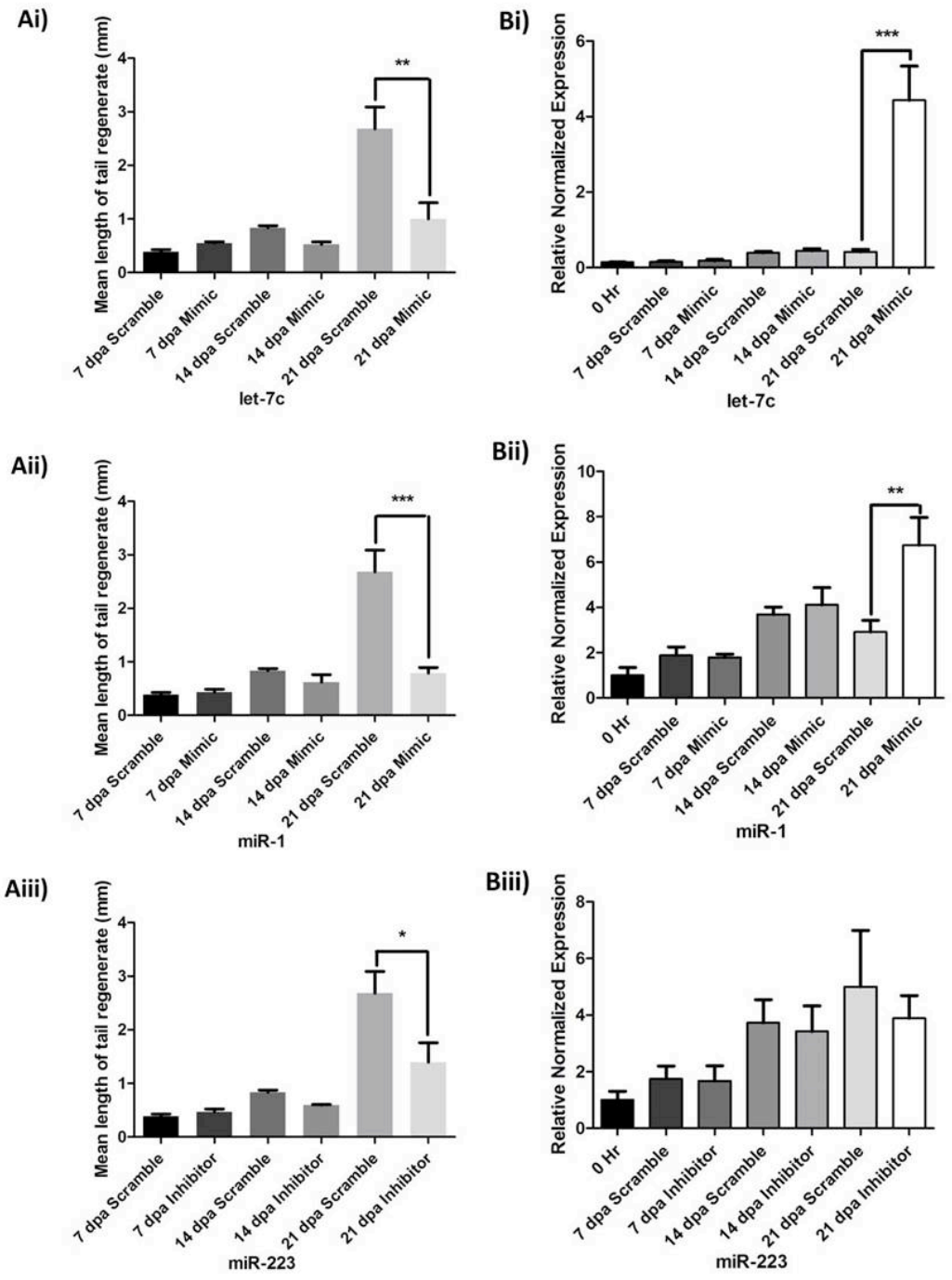


**Figure 3.8.** Graphical representation of mean regenerate tail lengths after *in vivo* injection and electroporation of (Ai) a let-7c mimic, and (Aii) a miR-1 mimic, (Aiii) a miR-223 inhibitor compared to regenerate lengths after injection of a scrambled oligonucleotide. Error bars indicate standard error. B) Confirmation of mimic-induced upregulation of let-7c and miR-1 by qPCR. (Bi) let-7c (Bii) miR-1 (Biii) miR-223, although the relative expression levels of miR-223 were consistently lower after injection of the miR-223 inhibitor than those from regenerates after injection of a scrambled oligonucleotide at all times points examined, these differences were not statistically significant. Error bars indicate standard error. * P<0.05, **P<0.01, ***P<0.001 (n=3).

Following injection and electroporation at these timepoints, upregulation of both let-7c and miR-1 caused a significant decrease ($p<0.001$ for miR-1, $p<0.01$ for let-7c) in tail regenerate length by 21 dpa, compared to regenerates in animals receiving equivalent injections of a scrambled oligonucleotide (Figs. 3.7A, 3.7B, 3.7D, 3.8Ai, 3.8Aii). Similarly, injection of the miR-223 inhibitor led to a significant ($p<0.05$) decrease in the length of tail regenerates at 21 dpa compared to those animals receiving the scrambled oligonucleotide (Figs. 3.7C, 3.7D, 3.8Aiii). *In vivo* alterations of target miRNAs at each timepoint after injection and electroporation of each mimic or inhibitor were confirmed by qPCR (Fig. 3.8B). Thus, it appears that the downregulation of miR-1 and let-7c after time 0, and the upregulation of miR-223 at day 7 previously observed by q-PCR and LNA-ISH, are required for normal regeneration of the spinal cord and tail.

*miR-1 targets the 3'-UTR of the newt RARβ2 mRNA in vitro.*

The temporal and spatial patterns of expression for miR-1 and miR-133a are very similar, with both being abundant within the ependymal layer of the unamputated tail followed by a significant downregulation within the same tissues by 14 dpa (Fig. 3.4) (Lepp and Carlone, 2014). We have recently demonstrated that miR-133a targets the 3'-UTR of *Notophthalmus viridescens* RARβ2 in an *in vitro* luciferase expression assay (Lepp and Carlone, 2014). Moreover, *in vivo* injection and electroporation of a miR-133a mimic led to a significant decline in RARβ2 levels by 14 dpa. This mimic-induced downregulation of RARβ2 in turn significantly inhibited tail and spinal cord regeneration (Lepp and Carlone, 2014).

Given these previous results, and the fact that miR-1 and miR-133a are contained within the same gene cluster (Wystub et al., 2013), and may be expressed coordinately,

our next objective was to determine whether miR-1 also targets RARβ2. Indeed, many messenger RNAs are targeted by multiple miRNAs, just as many miRNAs may target multiple messenger RNAs (Bhalala et al., 2013). A search of the miRanda database (microrna.org), and a multiple sequence alignment demonstrated that there are several potential binding sites for miR-1 on the previously cloned 3' UTR of the newt RARβ2 (Fig. 3.9) (Carter et al., 2011).

```
miR-1          ------------------------------------------------------------
RARβ2          GGCTCCCCAAGTCCAGATGAGGATACGACAGAGCACAGCCCCACCATCTCAACAAGCTCG 60

miR-1          ----------------------------------------------------ACCTT-- 5
RARβ2          GCAGACAACCGCAGCACTGGGGAATCTCCTCCAGTGAAATAGGAAGTTAATTGACTTTTT 120
                                                                  ** **

miR-1          ---ACATTT--------------CTTCATACATA-------------------------- 22
RARβ2          AAGACATTCATATTAAAAAAAAACCTTATACACAGCTAGGAAAACCCCTCATGGCTTGGT 180
                   *****               * * ***** *

miR-1          ---------
RARβ2          TTCCAGAAC 189
```

**Figure 3.9.** Newt RARβ2 mRNA contains three potential binding sites for miR-1. A sequence alignment of the 3'-UTR of the newt RARβ2 mRNA sequence with mature the mature *Danio rerio* miR-1 revealed three potential binding sites *. The first binding site includes sequences from the seed region of miR-1.

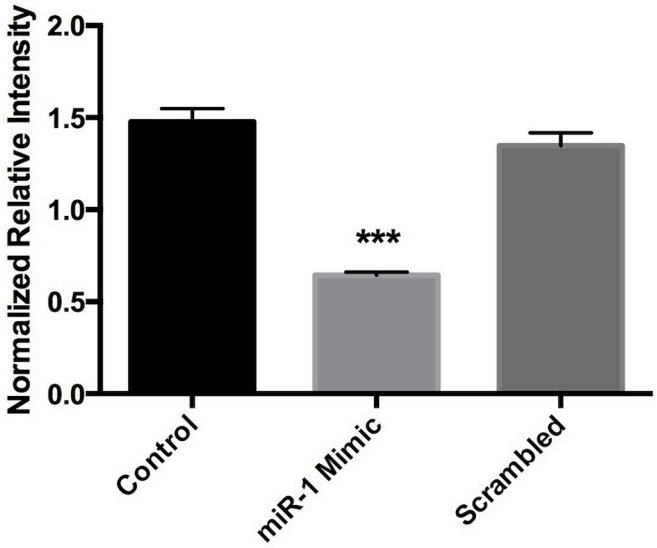


**Figure 3.10.** The 3' UTR of newt RARβ2 is a direct target of miR-1 *in vitro*. The pmiR-Glo luciferase reporter assay verified direct binding of miR-1 with putative binding sites on the 3'-UTR of newt RARβ2 mRNA after normalization to the internal *Renilla* luciferase activity. Control= Relative luciferase activity from the pmiR-Glo plasmid in PC3 cells containing the newt RARβ2 3'-UTR in the absence of exogenous miR-1. The addition of the miR-1 mimic to the cells led to a 56% decline in luciferase activity while the addition of a scrambled nucleotide had no significant effect. This decline in activity was significantly different from both the control and scramble-treated cells (***P< 0.001 in comparison to the control and scrambled)(n=12 for each condition).

Our first step in determining whether RARβ2 is a target of miR-1 was to test the ability of miR-1 to bind to and inhibit expression of a reporter gene containing the 3' UTR of newt RARβ2. Human PC3 cells were transfected with the pmirGLO Dual Luciferase plasmid containing the newt RARβ2-3'UTR downstream of the firefly luciferase gene, and co-transfected with the miR-1 mimic (used in the functional studies above) or a scrambled oligonucleotide (as a control). Co-transfection of the reporter plasmid with the miR-1 mimic caused a significant decrease in firefly luciferase activity (Fig. 3.10), compared to the control cultures containing the reporter plasmid but without the miR-1 mimic (56% reduction, $p<0.001$). There was no significant difference in normalized firefly luciferase activity between cultures co-transfected with plasmid alone and those cultured with plasmid and scrambled oligonucleotide (8% reduction, $p<0.219$, Fig. 3.10). Thus, miR-1 binds to the 3'-UTR of the newt RARβ2, leading to translational inhibition *in vitro*.

To determine if miR-1 targets the newt RARβ2 mRNA *in vivo*, we examined the effect of miR-1 mimic injection on the levels of RARβ2 protein in tail regenerates using a Western Blot analysis (Fig. 3.11A). Mimic-based upregulation of miR-1 caused a significant decrease in RARβ2 levels at 7 dpa ($p<0.05$), 14 dpa ($p<0.05$) and 21 dpa ($p<0.01$) compared to regenerates receiving the scrambled oligonucleotide (Fig. 3.11B).

These data support a role for both miR-1 and miR-133a in the translational regulation of RARβ2 protein in the regenerating adult newt tail. Elevated levels of both miRNAs in the unamputated tail may function coordinately to suppress RARβ2 expression in the ependymal cells of the intact spinal cord. Following injury, decreases in

the levels of these miRNAs by 14 dpa may thus contribute to an increase in the abundance and/or stability of mRNA transcripts encoding RARβ2. In turn, based upon our microarray data, this increase in RARβ2 may contribute to an ongoing suppression of miR-1 and miR-133a expression, thus maintaining sufficient RARβ2 expression to promote ependymal cell proliferation, formation of the ependymal bulb and tail regeneration (Carter et al., 2011).

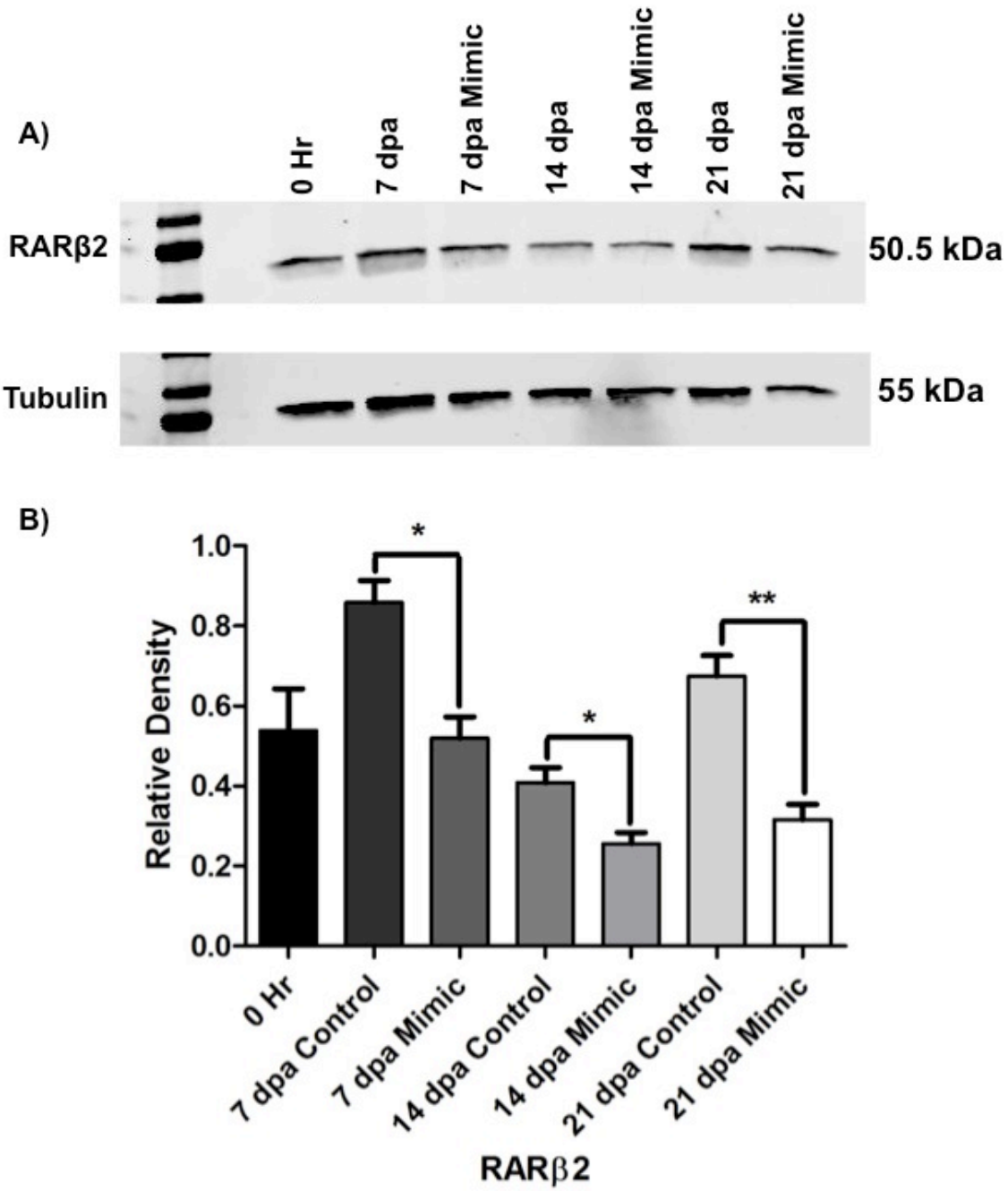


**Figure 3.11.** RARβ2 is a target of miR-1 in the newt during tail regeneration. A) Western blots of RARβ2 and tubulin proteins from tail regenerates at various time points after amputation and injection of either an miR-1 mimic or scrambled oligonucleotide. RARβ2 has an apparent M.W. of approximately 50.5 kDa, in confirmation of the previous results of Carter et al., (2011). B) Graphical representation of the relative densities shown in (A) determined by comparing the RARβ2 signal to the normalizing control, tubulin. Levels of RARβ2 were significantly decreased in mimic-treated regenerates at 7, 14 and 21 dpa versus the control. Error bars represent standard error. *P<0.05, **P<0.01 (n=3 for each condition).

### 3.05 Discussion

*Inhibition of retinoid signaling leads to dysregulation of numerous miRNAs in the regenerating newt tail.*

The cellular and molecular mechanisms that underlie the ability of a regeneration-competent species, such as the adult newt, to regenerate functional tissue after caudal spinal cord transection remain largely elusive. Recently, efforts to identify factors coordinating this complex epimorphic process have focused on the role played by miRNAs, since they have been shown to regulate complex signaling pathways by targeting multiple mRNAs for degradation in other systems. Several studies have in fact examined the role of miRNAs during epimorphic regeneration of a variety of tissues. These include the let-7 family of miRNAs in lens regeneration (Nakamura et al., 2010; Tsonis et al., 2007), miR-128 and miR-133 in cardiac regeneration in the newt (Witman et al., 2013) and zebrafish (Yin et al., 2012) and miR-21, miR-133a and miR-203 in limb and fin regeneration in the axolotl and zebrafish (Thatcher et al., 2008; Yin and Poss, 2008; Holman et al., 2012).

In response to tail amputation in the axolotl, miR-196 is upregulated, targeting *Pax-7* and *BMP4* to aid in the patterning of the regenerating spinal cord (Sehm et al., 2009). More recently, Diaz Quiroz et al., (2014) employing a microarray approach comparing miRNAs expressed in axolotl and rat spinal cords after transection, determined that precise levels of miR-125b within the ependymoglia cells post-injury are required for functional recovery. In this study, the authors identified *Sema4D* as a potential target for this miRNA *in vivo*. We did not detect miR-125b in our microarray as a microRNA dysregulated after inhibition of retinoid signaling through RARβ. However

we have recently determined that a related microRNA, miR-125a, is significantly downregulated after inhibition of retinoic acid synthesis with DEAB in the regenerating newt tail (unpublished). It will be of interest to determine the cell-type specific pattern of expression and putative target(s) of miR-125a in the newt.

The miR-133 family of miRNAs has also been shown to be involved in mediating gene expression post-transcriptionally during spinal cord regeneration. For example, Yu et al. (2011) found that upregulation of miR-133b promotes axonal outgrowth by targeting an inhibitor of this process, RhoA in the zebrafish. More recently, we have provided evidence that miR-133a downregulation in ependymoglia cells in the newt promotes caudal tail and spinal cord regeneration by maintaining retinoid signaling through RARβ2 in these cells (Lepp and Carlone, 2014).

The previous study represented the first to examine miRNA regulation of RARβ2-mediated retinoid signaling in any tissues during epimorphic regeneration in either vertebrates or invertebrates. None however, have attempted to determine the effects of retinoid signaling on the regulation of expression of miRNAs as target genes during this regenerative phenomenon. In the present study, we have utilized a microarray approach to identify at least 18 miRNAs that may represent either direct or indirect targets of RARβ2 signaling during caudal spinal cord regeneration in the newt. In confirmation of the microarray data, q-PCR analysis revealed that the expression of four of these miRNAs, miR-1, miR-26a, miR-223, and let-7c, was significantly altered at 14 days post tail amputation (dpa) after treatment with LE135, a selective RARβ antagonist. It is interesting to note that Carter et al., (2011) had previously shown that RARβ2 expression in ependymoglial cells of the newt spinal cord is significantly upregulated between 7 and

14 dpa. Thus is it not surprising that inhibition of retinoid signaling through that receptor subtype would have its greatest effect on potential downstream targets, including those miRNAs listed above, during this regenerative time period.

Each of the four miRNAs shown to be differentially expressed in response to RARβ inhibition at 14 dpa have been linked to the pleiotropic control of processes in other systems, but are also thought to contribute to the epimorphic regeneration of the newt tail and spinal cord. For example, in the mouse, during both development and in the adult CNS, miR-26a is expressed in astrocytes and neurons (Saugstad, 2010; Smirnova et al., 2005) and may function in neuronal differentiation by suppressing RNA polymerase II C-terminal domain small phosphatases (CTDSPs), which normally repress neuronal gene expression in neural stem cells (Dill et al., 2012). Thus the generation of mature miR-26a/b is required for neuronal differentiation. Zhu et al. (2012) have shown that miR-26 a/b inhibits cell proliferation in mouse primary fibroblasts by targeting CDK6 and cyclin E1, leading to a decrease in the phosphorylated form of pRb protein, thus blocking the G1/S transition. In our study, miR-26a levels are initially high in the unamputated tail and then decrease by 14 dpa and begin to rise again by 21 dpa. This pattern is consistent with a role for this miRNA in maintaining the differentiated state in the unamputated tail, and, in response to increased expression of RARβ2 after tail amputation, its downregulation provides an environment permissive for cellular proliferation and maintenance of the neuronal stem cell state (or as cells with transit-amplifying characteristics, Kirkham et al., 2014) as the ependymoglial outgrowth continues. Later on in the regeneration process, the observed increased expression of miR-26a could be associated with the onset of neuronal, myogenic or osteogenic

differentiation as witnessed in human cord blood unrestricted stem cells (Trompeter et al., 2013).

We saw a similar pattern of expression to that of miR-26a with another putative RARβ2 downstream target, miR-145. Among the targets of miR-145 are superoxide dismutase-2 (SOD2) (Bhalala et al., 2013; Saugstad, 2010), and the anti-apoptotic factor, Bcl2 (Liu et al., 2009). Human ES cells express miR-145 after treatment with exogenous RA, suppressing expression of Oct4, Sox2 and Kruppel-like factor-4 (Klf4) (Jain et al., 2012). The precise role of this miRNA in the regeneration of the tail and spinal cord is unclear since both its cell-type specific pattern of expression and its putative target(s) in this system remain to be identified.

Expression of miR-223 was initially upregulated in response to tail amputation, with a more than 4 fold increase by 7 dpa compared to the levels measured in the unamputated time 0 tail and spinal cord. miR-223 levels then decreased significantly by 14 dpa and remained lower than those seen in the unamputated tail throughout the remainder of the regeneration period studied. miR-223 was initially identified due to its ability to modulate the differentiation of various hematopoietic lineages (Taïbi et al., 2014). In the mammalian SCI model, miR-223 has been linked to the early post-injury inflammatory response (Nakanishi et al., 2010; Strickland et al., 2011; Yunta et al., 2012), and has been shown to be present in neutrophils (Izumi et al., 2011), monocytes and macrophages (Taïbi et al., 2014). miR-223 has also been shown to regulate obesity-associated adipose tissue inflammation by targeting *Pknox1* and thus regulating macrophage polarization and enhancing alternative anti-inflammatory responses in mice (Zhuang et al., 2012). The critical role of macrophage infiltration has recently been

examined during the early phases of epimorphic regeneration of the axolotl limb (Godwin et al., 2013) and zebrafish caudal fin (Petrie et al., 2014). In the axolotl limb, simultaneous induction of inflammatory and anti-inflammatory markers appear within the first 24 hours pa (Godwin et al., 2013). Zukor et al., (2011) have also shown that macrophages are present by at least 14 days of spinal cord regeneration in the newt. These macrophages are often associated with regenerating axons. It would be interesting to determine whether miR-223 is expressed in newt macrophages and whether it targets the newt homolog of *Pknox1* in this complex, temporally regulated inflammatory/anti-inflammatory process. Since in the present study, miR-223 expression was found to be upregulated in ependymoglial cells at day 7, as determined by LNA *in situ* hybridization, it would also be critical, in future studies, to determine the interplay between retinoid signaling and miR-223 on the regulation of macrophage/ependymoglial interactions during the regeneration process.

In contrast to the upregulation of miR-223, both miR-1 and let-7c were consistently downregulated in response to injury during the first 21 dpa. As with miR-223, the expression of both of these microRNAs was prominent within ependymoglial cells and their relative abundance in these cells reflected the tissue levels as determined by q-PCR at similar regeneration stages. The fact that these three miRNAs are expressed in the same tissues and at similar regenerative stages as RARβ2 (Carter et al., 2011) is significant and supports our microarray data demonstrating either a direct or indirect effect on expression levels of these microRNAs after inhibition of RARβ signaling. Additional support comes from *in vitro* studies on embryonic stem cells (ESCs)/smooth muscle cells (SMCs) from mice, human peripheral blood, and human primary blast cells,

demonstrating that addition of RA alters the expression of these three miRNAs (Fazi et al., 2005; Huang et al., 2010; Saumet et al., 2009; Xie et al., 2011; Zardo et al., 2012).

The precise roles of miR-1 and let-7c as targets of RARβ signaling during spinal cord regeneration remains to be determined. Let-7c has been shown to be involved in mouse ESC renewal (Melton et al., 2010) and its expression can be induced by exogenous addition of RA to human NB-4 cells (Saumet et al., 2009). Let-7 family members are downregulated during the process of dedifferentiation in the dorsal iris pigmented epithelial cells (PECs) after lentectomy in the adult newt (Tsonis et al., 2007). More recently, gain and loss-of-function analyses have provided evidence that up-regulation of let-7b controls proliferation of both dorsal and ventral PECs in the regenerating newt lens (Nakamura et al., 2010). Our results indicate that let-7c expression is also downregulated by 7 dpa within the ependymal layer of the regenerating spinal cord and is maintained at a low level in these cells for the remaining two weeks of our analysis. This downregulation may be consistent, at least initially, with a role for let-7c in this system to maintain cells in a stem cell-like proliferative state within the ependymal tube. Further studies are required to determine its precise function and specific target mRNAs in these cell types during the regeneration process.

In mammalian models of spinal cord injury, miR-1 has been shown to contribute to an anti-inflammatory reaction by targeting annexin (Bhalala et al., 2013; Liu et al., 2009), as well as to induce apoptosis (Strickland et al., 2011). *In vitro*, both miR-1 and miR-133a/b regulate the myoblast-to-myocyte differentiation process by targeting myogenic differentiation antigen-1 and myogenin among others (van Rooij et al., 2008). Georgantas et al., (2014) have recently proposed that cytokine signaling may inhibit

myoblast differentiation by the suppression of miRs-1, 133a and 206, thus providing a link between the inflammatory response and muscle degeneration characteristic of the early phases of tail regeneration. In the present study however, we restricted our analysis of the expression of these microRNAs to those events occurring after 7 days post injury, a time when muscle degeneration in the tail has subsided and blastema mesenchyme proliferation is prominent distal to the wound site (Butler and Ward, 1967; Chernoff et al., 2003; Diaz Quiroz and Echeverri, 2013; Iten and Bryant, 1976). miR-1 expression is lowest at 14 dpa in our study, at a time when RARβ2 expression is upregulated compared to time 0 (Carter et al., 2011). At this regeneration stage, the ependymal tube extends distally within the blastema as both ependymoglial and blastema cells continue to proliferate. The basement membrane within the proximal portion of the epidermis has begun to form at this point, and a cartilaginous rod extends beneath the ependymal tube, providing support to it and regenerating axons (Iten and Bryant, 1976).

Although each of these miRNAs may have multiple targets within the newt during tail and caudal spinal cord regeneration, including the ones suggested above, we were interested in determining whether any of our identified miRNAs had components of the retinoid signaling pathway as potential targets. Since both miR-1 and retinoic acid signaling have been widely reported to be important regulators of inflammation, apoptosis and muscle degeneration in mammalian tissues (Bak et al., 2008; Bhalala et al., 2013; Saugstad, 2010; Strickland et al., 2011), and since their expression patterns are coincident spatially but inversely correlated temporally, we decided to focus our attention on RARβ2 as a potential target of miR-1. Although scant evidence exists that miRNAs target RARs (Nervi and Grignani, 2014), an *in silico* analysis in miR-base (TargetScan

Human 6.2) revealed that several miRNAs, including miR-1 and miR-133a, are complementary to sites within the 3'-UTR of the RARβ mRNA transcript. We have previously provided evidence that miR-133a targets newt RARβ2 both *in vitro* and *in vivo* (Lepp and Carlone, 2014). Furthermore, miR-1 and miR-133a are encoded within the same gene cluster and may be regulated in a coordinated fashion (Kusakabe et al., 2013) as they appear to be in the present study. Both miR-1 and miR-133a are significantly more abundant in uninjured spinal cord tissue compared to the levels seen in ependymal cells after 7 dpa. Conversely, as mentioned above, RARβ2 expression increases significantly above the levels in unamputated spinal cord tissue by 7 dpa (Carter et al., 2011). Taken together, these data are suggestive of a negative feedback loop in which miR-1 and miR-133a are expressed in ependymoglial cells in the unamputated tail to contribute to the suppression of RARβ2 expression (Fig. 3.12). In response to injury, concomitant with an increase in RARβ2 in ependymoglial cells, miR-1 and miR-133a

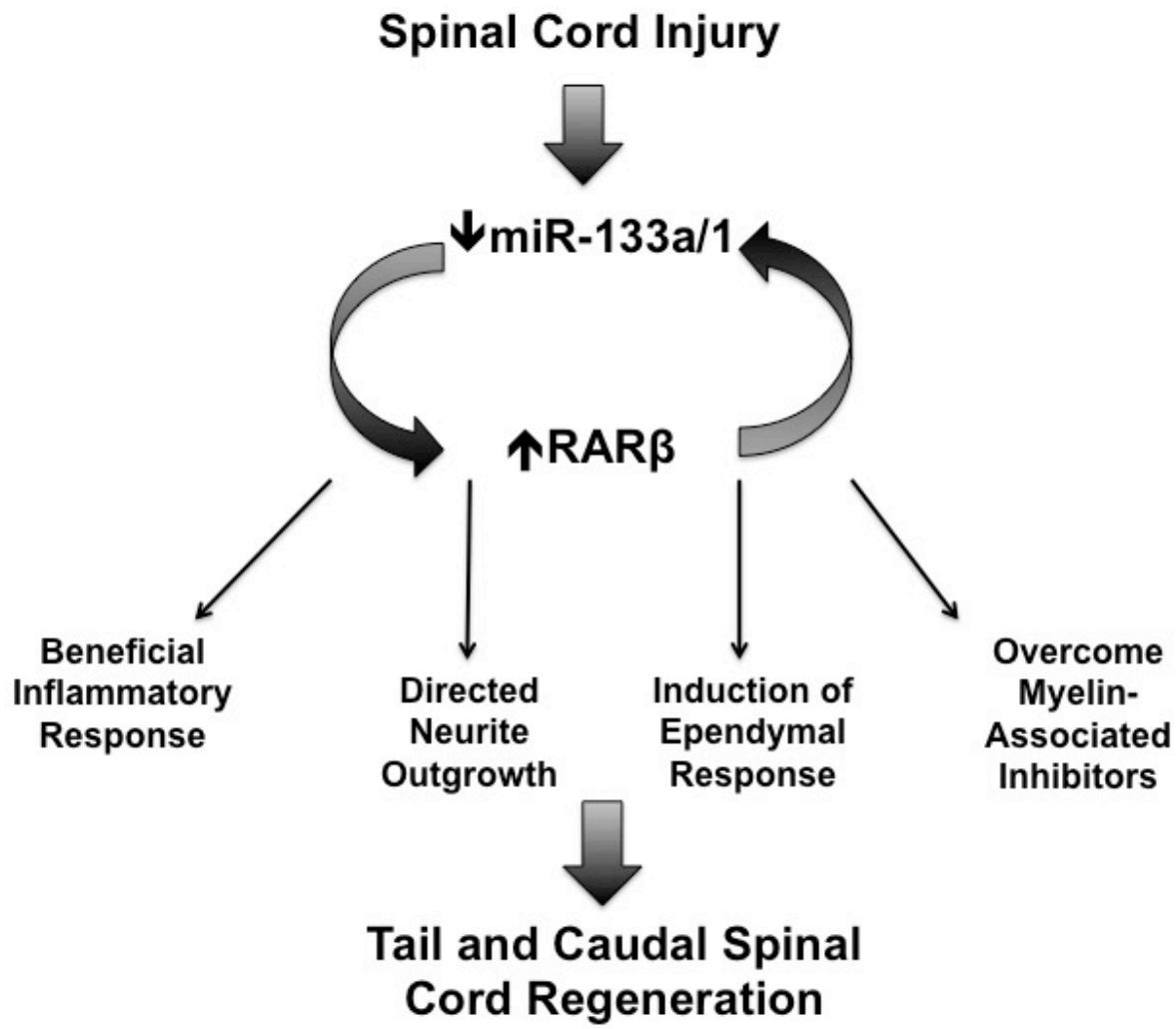


**Figure 3.12.** Potential model depicting the involvement of miR-133a, miR-1 and RARβ2 during tail and caudal spinal cord regeneration in the adult newt. In response to injury, miR-133a and miR-1 are downregulated by an unknown factor, which allows for an increase in RARβ2 expression. This forms a putative negative feedback loop to maintain the upregulated RARβ2, which in turn may be involved in the activation of the ependymal response, inflammation, and contribute to the establishment of a permissive environment for regeneration.

levels diminish significantly. Since our data strongly support the 3'-UTR of RARβ2 as a target for both miR-1 (this study) as well as miR-133a *in vitro* and *in vivo*, (Lepp and Carlone, 2014), their downregulation could contribute to the maintenance of RARβ2 signaling in the ependymal layer of the cord which is essential for continual ependymal outgrowth, neuronal differentiation and ultimately tail regeneration (Carter et al., 2011). These data represent the first evidence in support of a negative feedback loop, albeit indirectly, between retinoid signaling and miRNAs in spinal cord regeneration in a regeneration-competent model organism. Future studies are aimed at determining whether any of the miRNAs that we have identified have retinoic response elements (RAREs) as part of their gene regulatory sequences or whether other effector molecules mediate the effects of RARβ2 signaling on the expression of these miRNAs indirectly.

**3.06 Acknowledgements**

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# CHAPTER FOUR:

## Inhibition of retinoic acid synthesis promotes microRNA dysregulation during caudal spinal cord regeneration in the adult newt.

**4.01 Abstract**

Retinoic acid signaling is involved in the patterning and differentiation of the central nervous system, the maintenance of the neuronal differentiated state after development, and is actively involved in regeneration of the nervous system in the adult (Blum and Begemann, 2013; Maden, 2007). Inhibition of retinoic acid synthesis during development leads to deficits in spinal cord and hindbrain formation in mammalian embryos (Duester, 2008). However the effects of retinoic acid synthesis inhibition during spinal cord regeneration in a species capable of recovery from a spinal cord injury (SCI) remain unknown. We have previously shown that inhibition of retinoid signaling via the retinoic acid receptor, RARβ, during caudal spinal cord regeneration in the adult newt leads to microRNA dysregulation. That study only examined those microRNAs affected by a decline in retinoid signaling through RARβ, and thus potentially excluded microRNAs regulated by other retinoid X or RAR type receptors. Our main objective for this study was to examine the effect of inhibiting retinoic acid synthesis on microRNA expression during caudal spinal cord and tail regeneration in the adult newt. Using a microarray, we identified 4,426 miRNAs expressed in two treatment groups, treatments with either the retinaldehyde dehydrogenase inhibitor diethylaminobenzaldehyde (DEAB), or with the vehicle control, DMSO. Of these miRNAs, 367 had statistically significant differential expression between the treatment groups, and only 61 of these miRNAs had high relative signal intensities (>500) in the treatment, control, or both groups. Six of these miRNAs were chosen for further profiling based on known roles in either inflammation, apoptosis or protection against oxidative damage. RT-qPCR analyses of these six miRNAs confirmed dysregulated expression in response to retinoid

synthesis inhibition by DEAB treatment over the first two weeks post amputation. These data provide the framework for future studies to uncover the potential roles of each of these miRNAs. A more thorough understanding of which miRNAs are regulated by the retinoid signaling pathway during regeneration, as well as their targets and downstream functions may contribute to unraveling the complex pathways that provide a permissive environment for tail regeneration in the newt.

## 4.02 Introduction

Retinoid signaling has been implicated in regeneration and recovery from a spinal cord injury, where the re-activation of processes from development such as stem cell differentiation, axonal outgrowth and patterning to re-establish functional connections are critical. For example, spinal cord explants from mouse embryos exhibit neurite outgrowth in response to RA treatment *in vitro*, but this response is absent in adult spinal cord explants (Corcoran et al., 2002). Although adult mouse spinal cord explants showed no neurite outgrowth in response to treatment with RA, when transduced with a lentiviral vector containing the gene for RARβ2, outgrowth occurred *in vitro* similar to that seen with the cultured embryonic explants (Corcoran et al., 2002). Furthermore, neurons treated with a selective agonist of RARβ2 (CD2019) are capable of neurite outgrowth *in vitro* through an inhibitory environment containing myelin-associated glycoprotein (MAG) (Agudo et al., 2010). These data suggest that a lack of RARβ2 expression in the adult CNS may be responsible for the lack of neurite outgrowth observed in mammals after a SCI.

Species capable of regenerating spinal cords after a SCI, such as the adult newt, exhibit neurite outgrowth *in vitro* from spinal cord explants treated with RA, and this effect is specifically mediated by RARβ (Dmetrichuk et al., 2005). These animals not only have RARβ2 expression throughout the CNS into adulthood, but RARβ2 expression is upregulated in response to caudal tail amputation (Carter et al., 2011). Inhibition of RARβ2 signaling in caudal spinal cords after tail amputation by a specific antagonist, LE135, leads to inhibition of tail and spinal cord regeneration (Carter et al., 2011).

The effectors of RARβ2-mediated retinoid signaling, which contribute to functional tail and spinal cord regeneration, are just beginning to be uncovered. We have previously demonstrated that several miRNAs are not only acting downstream of RARβ2 signaling, but at least two miRNAs, miR-1 and miR-133a, may also target RARβ2 (See Chapters 2 and 3 of this thesis). However, focusing on RARβ2 alone does not provide a complete picture of retinoid signaling and miRNA involvement in this pathway during tail regeneration. While five RAR isoforms have been identified in regenerating newt tissue (Carter et al., 2011; Maden and Hind, 2003), RARβ is the only one of these receptor subtypes currently demonstrated to be involved in regeneration (Carter et al., 2011; Dmetrichuk et al., 2005). Retinoids can act through numerous receptor heterodimers, thus by focusing solely on miRNAs downstream of RARβ2 numerous miRNAs may be overlooked. It is possible that retinoid signaling is playing multiple roles during regeneration, and could also be involved in inflammation and stem cell maintenance. Our main objective here was to determine which, if any, miRNAs were affected by the inhibition of retinoid signaling at one of the first steps in the signaling pathway, the synthesis of the ligand all trans RA. By inhibiting the activity of retinaldehyde dehydrogenase (RALDH) and thus the synthesis of RA from retinaldehyde, we should be able to determine a more precise and total picture of the role of miRNAs and RA signaling in spinal cord regeneration in the newt.

## 4.03 Materials and Methods

*Animal Care and Surgery*

Adult eastern spotted newts, *Notophthalmus viridescens*, were used for all experiments and were purchased from Boreal Scientific (St. Catharines, ON). For the duration of the study, newts were housed in plastic containers in dechlorinated water, and were fed brine shrimp, bloodworms and liver three times a week. For surgical procedures, animals were anaesthetized by bathing for 10 minutes in 0.1% tricaine methane sulfonate (MS-222, Sigma), pH 7.0. The tails of anaesthetized animals were amputated approximately 1 cm caudal to the cloaca, after which they recovered on ice for 20 minutes. Blastemas were collected at each timepoint by repeating the above anesthetic procedure, then transecting the regenerate tissue approximately 1-2 mm rostral to the original cut site. All animal care and surgery procedures were approved by the Brock University Animal Care and Use Committee.

*DEAB Treatment*

Immediately after recovery post surgery, animals were placed in a bath containing dechlorinated tap water and either $10^{-6}$ M diethylaminobenzaldehyde (DEAB), an inhibitor of retinaldehyde dehydrogenase (RALDH), or 0.01% DMSO (the vehicle control) for up to 14 days. This concentration was successfully used via bath application to completely inhibit RALDH activity in zebrafish embryos by Neto et al., (2012). Solutions were changed twice a week and newts were fed and maintained following the above housing protocol.

*Microarray Analysis*

Blastemas were isolated and immediately flash frozen from regenerates 4 days post amputation (dpa) and after treatment with either DEAB or DMSO. Total RNA was isolated from homogenized blastemas in each treatment group using the Animal Tissue RNA Purification Kit (Norgen Biotek), RNA was quantified and purity assessed by a combination of gel electrophoresis and NanoDrop spectrophotometry (Thermo Scientific). Triplicate samples from each treatment were pooled, and the concentration of each was determined to ensure there was a minimum of 2 μg. Microarray analysis was outsourced to LC Sciences (Houston, TX). The samples were initially size-fractioned, then each treatment sample was hybridized to its own custom Cy3 chip containing 4,426 miRNA probes (known miRNAs of *Danio rerio*, *Xenopus tropicalis*, *Xenopus laevis*, *Homo sapiens*, *Mus musculus*), and 73 controls from miRBase 20. Hybridization was performed on the μParaflo®Microfluidic Biochip platform, which contains *in situ* synthesized probes in triplicate that are complementary for each target miRNA. Images were collected of the chips using a laser scanner (GenePix 4000B, Molecular Device), signals were background subtracted and normalized using a LOWESS filter (locally-weighted regression). Statistical analysis included a two-tailed Student's T test and was also performed by LC Sciences to compare the two treatment groups, and only signals that were significantly different between the two groups ($p<0.01$), were considered for further study.

*RT-qPCR*

Regenerate tissue containing up to 2 mm of proximal tail stump was acquired at the time points 0, 7 and 14 dpa, and immediately flash frozen in liquid nitrogen. RNA

was isolated as described above and cDNA was synthesized using gene specific stem-loop primers (Varkonyi-Gasic et al., 2007) with the SuperScript III Reverse Transcriptase Kit (Invitrogen) with 350 ng of total RNA. Real time qPCR was carried out on a CFX Connect Real-Time System (BioRad), using iQ SYBR Green Supermix (BioRad) in a 20 μl reaction (10 μl of iQ SYBR Supermix, 5 μl of RNase free water, 1 μl of forward and reverse primers and 3 μl of cDNA). Each reaction was run with three biological and three technical replicates on the following program; 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 55° for 15 sec and 72° for 45 sec, then each reaction ended with a melt curve analysis. Following normalization to two reference genes (α-Tubulin, β-Actin), the $\Delta\Delta C_T$ method was used to determine the relative expression changes of each miRNA for each treatment and timepoint. The Minimum Information for Publication of Quantitative Real-Time qPCR Experiments (MIQE) guidelines were followed for all RT-qPCR experiments (Taylor et al., 2010). The following primers were used for stem-loop cDNA synthesis (RT primers), as well as qPCR:

| | |
|---|---|
| RT-miR-125a | gtcgtatccagtgcagggtccgaggtattcgcactggatacgaccacagg |
| RT-miR-21-5p | gtcgtatccagtgcagggtccgaggtattcgcactggatacgacctggaag |
| RT-miR-149-3p | gtcgtatccagtgcagggtccgaggtattcgcactggatacgaccacc |
| RT-miR-23a | gtcgtatccagtgcagggtccgaggtattcgcactggatacgacggaa |
| RT-miR-205 | gtcgtatccagtgcagggtccgaggtattcgcactggatacgaccagac |
| RT-miR-206 | gtcgtatccagtgcagggtccgaggtattcgcactggatacgacccaca |
| dre-miR-125a | cgctgggagagacccttaa |
| has-miR-21-5p | gccgcagtgtagtatgggca |
| mmu-miR-149-3p | gcgagggagggacgggg |
| xla-miR-23a | gcatcacattgccagggau |
| dre-miR-205 | cgctccttcattccaccg |
| dre-miR-206 | gccgctggaatgtaaggaag |
| has-miR21-5p 2 | cgcagtgtagtatgggca |
| Reverse | gtgcagggtccgaggt |

*Statistical Analysis*

Data for the miRNA microarray chip repeats were analyzed using a Student's two-tailed t-test ($p<0.01$). A one-way ANOVA with a Post Hoc Tukey test ($p<0.05$) was used to analyze all qPCR data. Unless otherwise indicated, all analyses were run in triplicate.

**4.04 Results**

*DEAB treatment inhibits tail and caudal spinal cord regeneration.*

It has previously been demonstrated that LE135, an RARβ-selective antagonist, inhibits normal caudal regeneration of the newt tail after transection (Carter et al., 2011), and leads to dysregulation of miRNA expression in response to injury (Chapter 3). However, since RARβ represents one of a number of receptor subtypes which may mediate RA's effects, my aim in this study was to determine the possible effects of inhibiting RA synthesis *in vivo* on miRNA expression during tail regeneration. To accomplish this, immediately after recovery from surgery at time 0, newts were placed in a $10^{-6}$ M solution of either DEAB, a potent inhibitor of the enzyme RALDH, or DMSO (vehicle control). Bath application of DEAB significantly inhibited normal tail regeneration, in some cases so severely that the tail tissues actually retracted rostrally away from the site of injury and the vertebral column was visible at the stump of the tail (Fig. 4.1B). Significant inhibition of tail regeneration was observed at both 14 and 21 dpa, but not at 7 dpa (Fig. 4.1C). This is not surprising, as less than 1 mm of tissue regeneration had occurred in both control and treatment groups (0.5683 mm in DMSO, 0.5137 mm in DEAB) so a significant difference would be very difficult to observe. However this does not mean that DEAB was not causing changes in early regeneration at the molecular level. It is clear that retinoid signaling is required for tail regeneration, a result in agreement with previous research which showed that LE135 treatment had a similar, albeit less severe effect (Carter et al., 2011). The severity of regeneration inhibition observed here is not surprising given that multiple pathways could be affected by the inhibition of retinoid signaling.

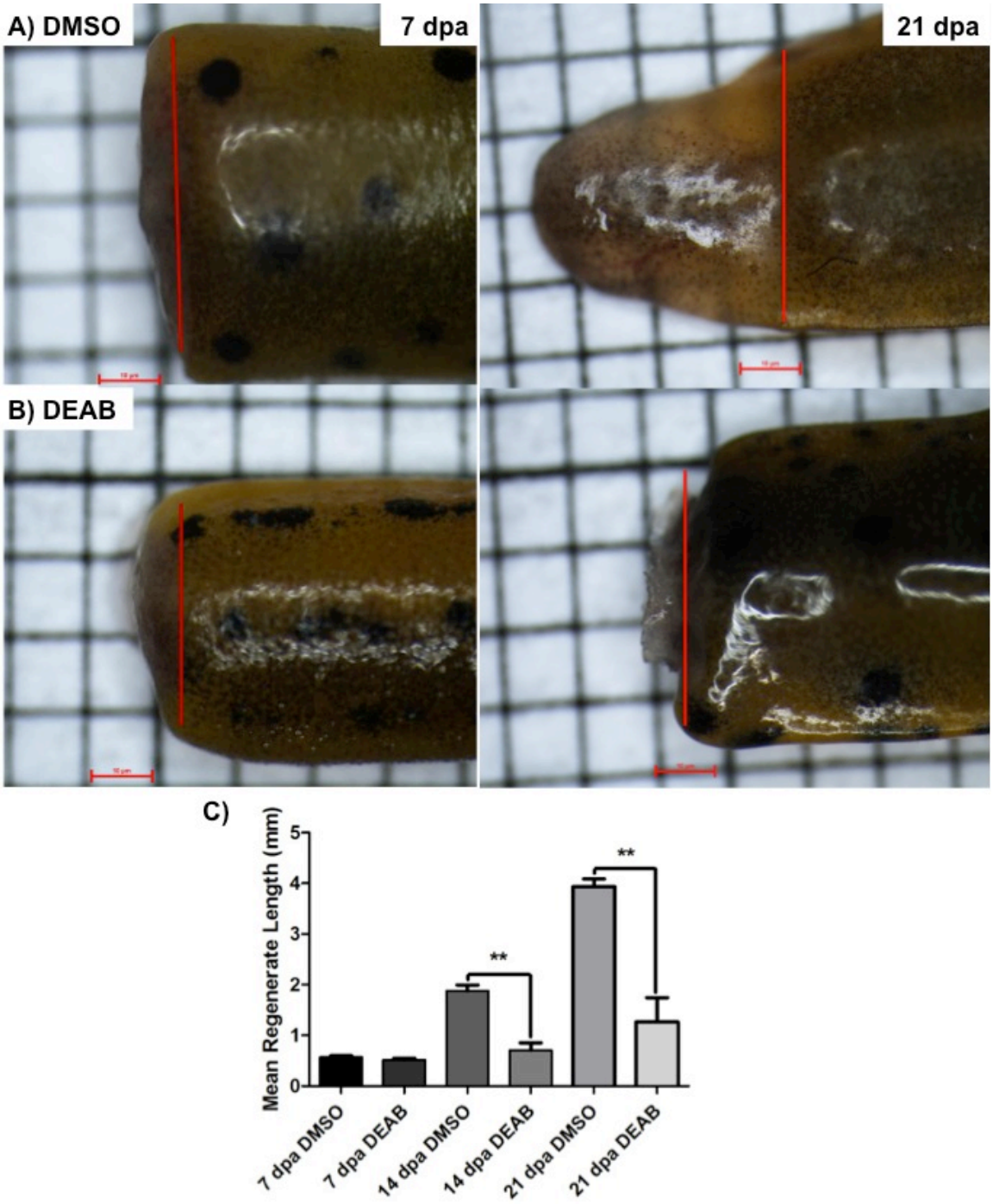


**Figure 4.1.** Bath application of DEAB, an inhibitor of the enzyme RALDH, inhibits tail regeneration. DEAB had no significant effect on tail regenerate length or blastema formation at day 7 pa compared to DMSO treatment (A, B). However by 14 and 21 days pa, DEAB significantly inhibited caudal regeneration (A, B, C). Error bars indicate standard error, ** $P<0.01$ (n=3). Scale bars = 1 mm.

*Treatment with DEAB causes widespread miRNA dysregulation early in regeneration.*

Our next specific question was to determine which miRNAs are affected by inhibition of RA synthesis. We have previously demonstrated that 18 miRNAs are affected by inhibition of RARβ signaling in tail regenerates (Chapter 3). We outsourced a microarray to LC Sciences (Houston, TX) with pooled total RNA from day 4 regenerates treated with either DEAB or DMSO, as explained above. miRNA expression was detected for each of the 4,426 probes tested, of which 367 miRNAs were significantly different between groups ($p<0.01$). A relative signal intensity above 35 is considered a positive test sample by LC Sciences, however for the purposes of this study we decided to focus only on reporter probes that had high signal intensity (>500) in the control, treatment, or both group's chips. This narrowed down the list of 367 to just 61 miRNAs to focus on for further examination (Table 4.1). Notably, miR-1, let-7c and miR-133a, were all on the "high expression" list, and were also the focus of my previous research (Chapters 2 & 3).

**Table 4.1.** Reporter probes which detected high miRNA expression, a signal intensity greater than 500, on the microarray in the control, treatment, or both groups at 4 dpa. The value given indicates the signal intensity for that tested group, and those in bold indicate significant differential expression between treatment and control groups. Filled boxes indicate the miRNAs chosen for further study. Reporter probes included dre (*Danio rerio*), xla (*Xenopus laevis*), xtr (*Xenopus tropicalis*), hsa (*Homo sapiens*) and mmu (*Mus musculus*).

| Reporter Name (miRNA) | DMSO Signal Intensity | DEAB Signal Intensity |
|---|---|---|
| **dre-miR-1** | **1,307** | **800** |
| **dre-miR-125a** | **1,500** | **709** |
| **dre-miR-125c** | **620** | **236** |
| **mmu-miR-149-3p** | **725** | **904** |
| **dre-miR-206** | **1,156** | **188** |
| **dre-miR-214** | **538** | **276** |
| **xtr-miR-26** | **241** | **670** |
| **dre-miR-26a** | **244** | **622** |
| **has-miR-3141** | **391** | **641** |
| **mmu-miR-341-5p** | **42,435** | **55,555** |
| **has-miR-4690-5p** | **1,765** | **2,621** |
| **has-miR-5096** | **282** | **1,186** |
| **mmu-miR-5112** | **26** | **1,260** |
| **mmu-miR-5126** | **744** | **1,127** |
| **mmu-miR-7028-5p** | **1,432** | **13,379** |
| **mmu-miR-705** | **419** | **542** |
| **mmu-miR-709** | **297** | **793** |
| **mmu-miR-7082-5p** | **298** | **925** |
| **mmu-miR-7116-5p** | **353** | **20,643** |
| dre-let-7a | 2,097 | 1,454 |
| dre-let-7b | 662 | 585 |
| dre-let-7c | 1,412 | 1,088 |
| has-let-7d-5p | 1,694 | 1,153 |
| xtr-let-7e | 1,186 | 667 |
| has-let-7e-5p | 621 | 526 |
| dre-let-7f | 1,369 | 978 |
| xtr-miR-1a | 1,105 | 643 |
| has-miR-125a-5p | 597 | 276 |
| xla-miR-133a | 682 | 112 |
| dre-miR-133a-3p | 880 | 221 |
| xla-miR-133b | 697 | 181 |
| dre-miR-133b-3p | 794 | 212 |
| mmu-miR-133b-5p | 49,751 | 39,048 |

| | | |
|---|---|---|
| xtr-miR-133c | 986 | 227 |
| xla-miR-133d | 587 | 152 |
| dre-miR-203a | 702 | 1,053 |
| has-miR-203a | 371 | 746 |
| dre-miR-203b-3p | 417 | 742 |
| dre-miR-205 | 561 | 288 |
| has-miR-21-5p | 736 | 586 |
| xla-miR-23a | 558 | 489 |
| xtr-miR-23b | 439 | 517 |
| mmu-miR-3064-3p | 519 | 390 |
| has-miR-3591-3p | 554 | 375 |
| has-miR-3613-3p | 20,072 | 15,496 |
| has-miR-3960 | 333 | 670 |
| has-miR-4267 | 3,489 | 961 |
| has-miR-4324 | 640 | 179 |
| mmu-miR-434-3p | 343 | 578 |
| has-miR-4459 | 551 | 803 |
| has-miR-466 | 627 | 545 |
| has-miR-4668-5p | 14,697 | 6,281 |
| has-miR-4734 | 1,135 | 2,833 |
| has-miR-6087 | 3,489 | 3,728 |
| has-miR-6089 | 244 | 590 |
| has-miR-6090 | 518 | 990 |
| mmu-miR-6239 | 397 | 507 |
| mmu-miR-7081-5p | 4,308 | 1,135 |
| mmu-miR-7239-3p | 1,798 | 238 |
| has-miR-7847-3p | 232 | 503 |

*miRNAs are differentially regulated in response to retinoid signaling inhibition.*

Among the miRNAs identified in the microarray above, of particular interest due to roles in previous studies were miR-21-5p, miR-23, miR-125a, miR-149-3p, miR-205, miR-206, miR-434-3p and miR-709 (Table 4.1). High levels of expression were seen in both the control and treatment groups for miR-125a, miR-21-5p and miR-149-3p and all three have been previously shown to act as anti-apoptotic regulatory RNAs (Ding et al., 2013; Rebane and Akdis, 2013; Yunta et al., 2012). miR-125a and miR-21-5p have also been associated with anti-inflammatory properties (Strickland et al., 2011; Yunta et al., 2012). Expression was low (<500) after DEAB treatment but high in the DMSO control for miR-23, miR-205 and miR-206. miR-23 has previously been found to target *Fas,* an apoptotic factor that is involved in reactive oxygen species-mediated cell death (Lin et al., 2011). Thus miR-23 may promote protection from oxidative damage. During the development of breast cancer, miR-205 has been found to target phosphatase and tensin homolog (PTEN) and inhibit apoptosis (Sun et al., 2014). Finally, miR-206 has been shown to decrease proliferation in mouse skeletal muscle satellite cells by targeting the paired-box transcription factor Pax7 (Chen et al., 2010), and also target the anti-inflammatory *annexin2*, and free radical destroying superoxide dismutase (SOD1) in adult rats post-SCI (Liu et al., 2009).

miRNAs that are highly expressed after DEAB treatment but not after control DMSO treatment include miR-434-3p and miR-709. Expression of miR-434-3p is associated with an increase in neural stem cell (NSC) differentiation in brain derived rat neurons (Jovičić et al., 2013). miR-709 has been found to target the Brother of the

Regulator of Imprinted Sites (BORIS) (Tamminga et al., 2008), an important factor in regulation of DNA methylation.

Of these miRNAs, we chose to further investigate six that had a high signal from the microarray; miR-21-5p, miR-23, miR-125a, miR-149-3p, miR-205, miR-206. Each of these miRNAs were chosen because they have been previously implicated in other processes that are also involved in regeneration, including inflammation, apoptosis, and protection from oxidative damage (Ding et al., 2013; Lin et al., 2011; Yunta et al., 2012). Although only miR-125a, miR-149-3p and miR-206 had significant differences in signal between treatment groups in the microarray, miR-205, miR-21-5p and miR-23a were also chosen due to their demonstrated roles in regeneration-associated functions in other species. An expression profile for each of these miRNAs was established for each treatment group at time 0, 4, 7 and 14 dpa using RT-qPCR (Fig 4.2). With the exception of miR-149-3p, the pattern of expression of each of these miRNAs matched the expression profile observed at 4 dpa in our microarray. miR-21, miR-125a, miR-205 and miR-206 were upregulated in response to tail amputation (Fig. 4.1A, B, D, F). Specifically, miR-21 was significantly upregulated in DMSO control regenerates very early post amputation. Treatment with DEAB abolished this upregulation at 4 dpa, and expression was maintained at a constant level for the subsequent 10 days in both control and treatment groups. miR-125a and miR-205 were both upregulated in response to injury as well, and their expression remained high in the control DMSO regenerates for the first 7 days. Again, the level of expression was significantly decreased by DEAB treatment for these miRNAs, at days 4 and 14 for miR-125a and days 4 and 7 for miR-205.

Expression of miR-23 remained constant for the first 7 dpa, and a slight decrease in expression, although an insignificant one, was observed at 14 dpa (P = 0.2705) (Fig. 4.1C). There were no significant differences between the control and treatment groups for miR-23, which is not surprising considering there was no significant difference observed in the microarray either for this miRNA. miR-149-3p was downregulated compared to time 0 in both the control and treatment groups, but only significantly in the 14 dpa control regenerates (Fig. 4.2E). An interesting trend was that 4 out of 6 of the miRNAs examined were at least initially upregulated in response to injury, but by 14 dpa, all six of these miRNAs were either downregulated compared to time 0, or returned to time 0 levels of expression. We have previously seen miRNA dysregulation in response to inhibition of RARβ signaling at day 14, a stage at which RARβ2 levels are normally increased in ependymal cells during spinal cord regeneration. The putative targets of these miRNAs remain unknown, and studies are currently underway in our lab to examine not only the targets of these miRNAs, but also to examine their temporal and spatial expression patterns at later time points (21-35 dpa).

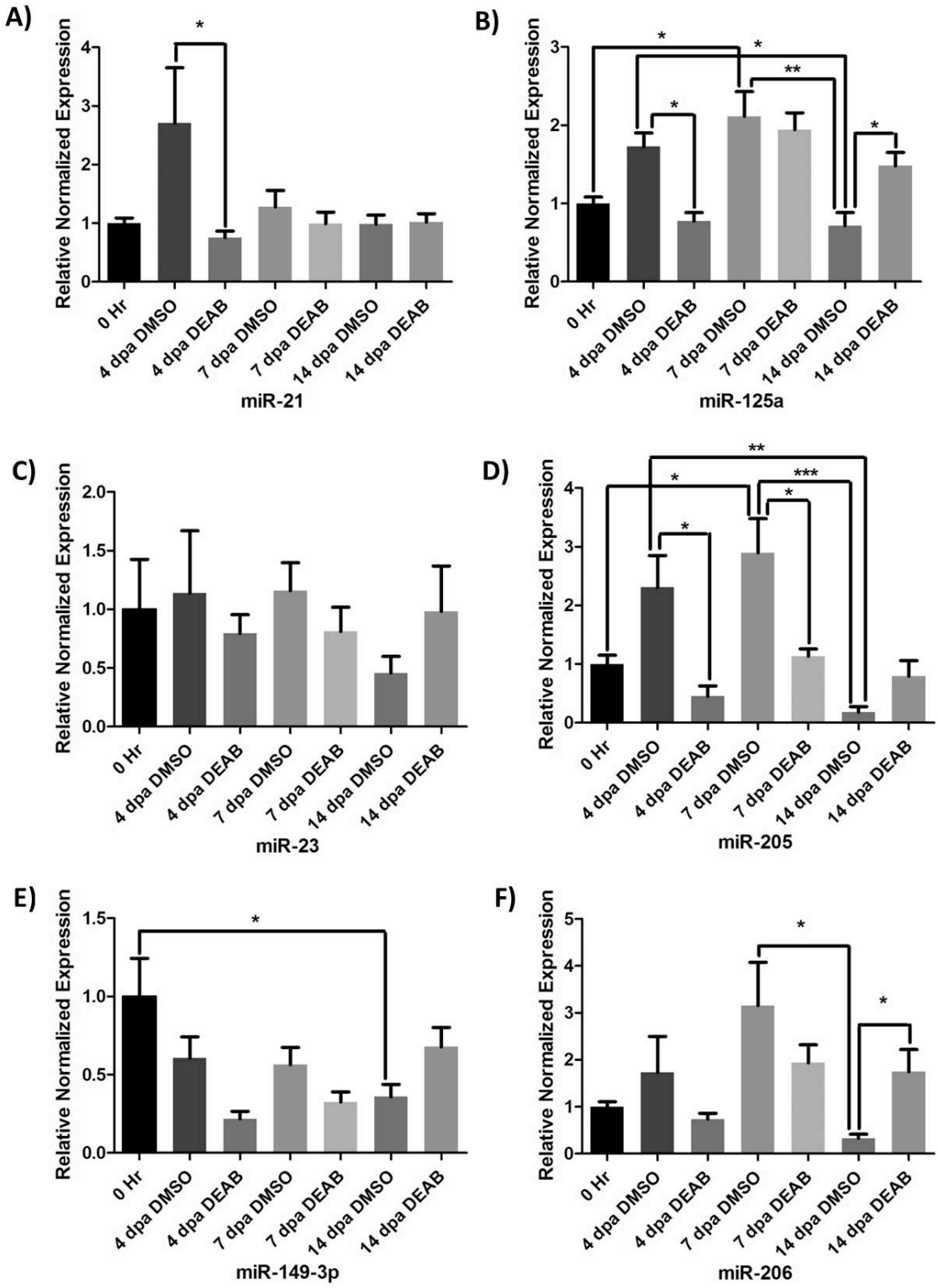


**Figure 4.2.** Relative normalized expression of six miRNAs determined by RT-qPCR in uninjured tissue and at three timepoints post amputation in regenerates from DEAB and control DMSO animals. A) miR-21 B) miR-125a C) miR-23 D) miR-205 E) miR-149-3p and F) miR-206. Error bars indicate standard error, * $P<0.05$ (n=3).

**4.05 Discussion**

The present study demonstrates, for the first time, that suppressing retinoic acid synthesis, via inhibition of retinaldehyde dehydrogenase (RALDH), leads to miRNA dysregulation as well as a reduction of tail and caudal spinal cord regeneration. While we provide evidence that treatment with DEAB significantly reduces the length of tail regenerates, the mechanism by which this decrease in RA acts on regeneration remains unclear. It is possible that the lack of regeneration observed can be attributed to interference with the epithelial-mesenchyme transition occurring during ependymal cell outgrowth, as retinoid signaling has been associated with this transition in neural crest cells (NCCs) during mouse development (Paschaki et al., 2012). Alternatively, retinoid signaling has also been associated with ependymal tube formation during urodele tail regeneration (Carter et al., 2011), as well as axonal guidance and neurite outgrowth in both mammals and urodeles *in vivo* and *in vitro* (Agudo et al., 2010; Corcoran et al., 2002, 2000; Dmetrichuk et al., 2005; So et al., 2006; Ping K Yip et al., 2006). Thus, the effects of inhibiting RALDH that we see here are likely pleiotropic, and lead to the overall inhibition of caudal tail and spinal cord regeneration.

Knock out experiments with mouse embryos have provided extensive phenotypic data demonstrating the consequences of RALDH inhibition on CNS development. Molotkova et al., (2007) found that *Raldh3 -/-* mouse embryos had a complete lack of neuronal differentiation in the developing forebrain. Furthermore, mice null for *Rdh10* also had defects in hindbrain patterning, as well as a lack of RA synthesis in the meninges (Chatzi et al., 2013). In *Raldh2 -/-* mouse embryos, where maternally supplemented RA is required to avoid lethality, the phenotype of the rescued embryos displays dorsal spinal

cord deficits, as well as inhibition of neuronal stem cell proliferation (Paschaki et al., 2012). Thus, retinoid signaling is a requirement for the normal development of the mammalian CNS, and deficiencies in RA due to mutations in RA synthesis genes leads to deficits in both brain and spinal cord formation.

Bath application of DEAB has also been used on zebrafish embryos to examine the effects of RA deficiencies on eye development (Le et al., 2012). Application of DEAB for just 2 hours on embryos that were 9 hours post fertilization was sufficient to cause microphthalmia that persisted for the remainder of development. Le et al., (2012) found that DEAB treatment effectively abolished RA synthesis, but did not cause immediate degradation of existing RA. Fin regeneration is inhibited in developing zebrafish larvae following treatment with DEAB, due to reduced mesenchymal proliferation (Blum and Begemann, 2013; Neto et al., 2012).

This is the first study to use any treatment of DEAB on the adult newt, and although it is likely that it is disrupting RA synthesis through RALDH inhibition due to its selectivity, further study is required to verify its specificity for RALDH2 in this species. The earliest tissue isolated in this study after beginning DEAB treatment was 4 dpa, which should provide ample time for any remaining RA to be degraded if its treatment is as potent as that observed by Le et al., (2012). In the present study I have not determined the effect of DEAB treatment on ependymal cell or tail blastema mesenchymal cell proliferation. Such a study could provide valuable information regarding the mechanism by which DEAB interferes with tail and caudal spinal cord regeneration in the newt.

In addition to demonstrating that inhibition of RA synthesis in the adult newt prevents tail regeneration, we also offer the first comprehensive profile of miRNA expression in response to inhibition of RA synthesis. Our microarray identified 4,426 miRNAs with varying levels of expression in response to DEAB treatment. The microarray we used is an established method for miRNA identification and is highly sensitive, with high probe quality as well as high stringency hybridization conditions. The enhanced detection sensitivity of this array, paired with the high level of conservation of miRNA sequences among distantly related species (Ha and Kim, 2014), provides confidence in the data generated by this microarray. Of the identified miRNAs, only 61 had high levels of expression in the control, treatment, or both groups (signal intensity >500). Many of these miRNAs, including but not limited to, miR-21-5p, miR-23, miR-125a, miR-149-3p, miR-205 and miR-206 have been previously implicated in other process that are involved in regeneration, including inflammation, apoptosis and protection from oxidative damage (Ding et al., 2013; Lin et al., 2011; Liu et al., 2009; Yunta et al., 2012). While only these six miRNAs were chosen for further analysis in this study, this microarray provides an excellent base for continued studies on numerous other miRNAs and their potential roles in retinoid signaling during spinal cord regeneration.

The differential expression of these six miRNAs which were identified with the microarray was then confirmed using qPCR, at various times during the first 14 days of regeneration in control and DEAB treated regenerates. As outlined in the results, each of these miRNAs displayed a different pattern of expression in response to injury, but just 4 out of the 6 miRNAs had significantly different expression between treatment groups. This result is not entirely surprising, as only 3 of the 6 miRNAs chosen had significant

differential signals detected in the microarray, the remaining 3 miRNAs were chosen solely based on their potential regulator-related roles in other systems. While these results are intriguing, the spatial pattern of expression, as well as putative target of each of these miRNAs remains to be determined. Thus, the precise role of each of these miRNAs during tail and spinal cord regeneration remains to be determined.

Previous research has provided evidence that miR-206 may be involved in recovery from both a muscular injury and SCI. Chen et al., (2010) identified a sharp downregulation of miR-206 in response to a cardiotoxin-induced muscle injury in mice, and found that it targeted *Pax7* in muscle satellite cells to repress proliferation. Alternatively, Liu et al., (2009) examined miR-206 expression following SCI in rats, which was initially downregulated in the first 24 hours post injury, but returned to baseline by 7 days post injury. Predicted targets of miR-206 in the rat were the anti-inflammatory factors *annexin A2* and *SOD-1*, as well as the anti-apoptotic factor *Bcl2-2* (Liu et al., 2009). Although miR-206 is downregulated in both of these mammalian studies in response to an injury, we observed the opposite in the newt in response to tail amputation. miR-206 was initially trending toward upregulation during the first 7 dpa (albeit not significantly, $p < 0.0798$), and treatment with DEAB prevented any change in expression. Thus the role of this miRNA remains elusive. However it is possible that it is involved in proliferation in response to injury in the newt as it is in mammalian satellite muscle cells.

An upregulation was also observed for miR-125a in response to injury; it was significantly upregulated at 4 and 7 dpa, but downregulated by day 14 in the control group. Little information exists on the role that miR-125a plays in response to SCI in any

system. However, Diaz Quiroz et al., (2014) recently examined the role of another miRNA from the same family, miR-125b. In response to a complete spinal cord transection in axolotls, miR-125b was downregulated in the radial glial cells surrounding the central canal of the spinal cord in the first 7 dpi. miR-125b was found to target *Sema4D*, and the precise expression pattern of miR-125b is required for the establishment of a permissive environment for axonal regeneration as well as lack of glial scar formation (Diaz Quiroz et al., 2014). The opposite pattern is seen in the present study for miR-125a, and although these miRNAs are related, it is possible that miR-125a has a completely different function in the newt in response to tail amputation. While the mature seed sequence of these two miRNAs is identical, there are seven bases that differ at the 3' end (miRbase.org). Considering that sister miRNAs often have completely different expression patterns, localization and targets (Ha and Kim, 2014), it is not completely surprising that a different pattern of expression was observed here.

In contrast to the upregulation seen with miR-206 and miR-125a, miR-149-3p demonstrated significantly lower expression at 14 dpa compared to time 0. miR-149-3p has previously been identified as an anti-apoptotic factor. In human blood it has been found to target *Puma*, a pro-apoptotic modulator (Ding et al., 2013). We observed a downregulation of miR-149-3p, so it is unlikely that it is targeting *Puma* in the newt because this would lead to an increase in apoptosis. However, this miRNA may still be involved in apoptosis, but likely has a different target.

Similar to miR-125a, miR-205 was significantly upregulated at both 4 and 7 dpa, but downregulated by day 14. *In silico* analysis suggests that miR-205 may target RARα (Nervi and Grignani, 2014). The expression pattern of RARα in the newt has not been

examined, but in the zebrafish, its expression is required for cardiomyocyte proliferation and heart regeneration (Blum and Begemann, 2013). No studies are available examining the role of RARα in tail or spinal cord regeneration in the newt. Future studies could examine not only its expression, but also whether it represents an *in vivo* miR-205 target.

Previous studies have also shown that miR-23 is upregulated in response to RA treatment in acute promyelocytic leukemia (APL) cells *in vitro* (Careccia et al., 2009; Saumet et al., 2009). The promoter region of miR-23 was found to have a promyelocytic leukemia (PML)-RARα binding site in these cells. The ability of RA to induce the expression of this miRNA remains unclear in the newt, as no significant difference in expression was observed in response to DEAB treatment. The potential target of this miRNA during regeneration also remains elusive.

Of particular interest was miR-21, which has previously been implicated as a modulator of the retinoid signaling pathway (Terao et al., 2011). In response to RA treatment in breast cancer cells *in vitro*, ligand-activated RARα leads to an upregulation of miR-21 in these cells, which in turn leads to an increase in proliferation (Terao et al., 2011). It has also been predicted to act as an anti-inflammatory and anti-apoptotic factor in response to SCI in mammals (Rebane and Akdis, 2013; Yunta et al., 2012).

In addition to miR-21 having previously been linked to retinoid signaling as well as inflammation and apoptosis, it has also been implicated during limb regeneration in the axolotl. In response to limb amputation, miR-21 is upregulated in the axolotl blastema but not stump tissue by 17 dpa, and was found to target *Jagged1*, an important patterning factor during development (Holman et al., 2012). We also found that miR-21 is upregulated very early during tail regeneration in the adult newt. More recent research

from our lab has shown that it is upregulated in untreated animals later during regeneration (days 28 and 35) (Rozema, 2014). Expression of *Jagged* mRNA was found to be quite high in uninjured tissue, and levels slowly decrease, with significant downregulation by days 28 and 35 (Rozema, 2014). Current studies are underway to confirm *Jagged* as a putative target of miR-21 in the newt tail during regeneration. Overall, the data presented here have laid the groundwork for many future studies examining miRNAs as key players in the regulation of the complex network of retinoid signaling components that are critical for tail regeneration in the newt.

**4.06 Acknowledgements**

Thank you to David Rozema for all of the help with sorting and analyzing microarray data as well as target prediction for this huge list of miRNAs.

# CHAPTER FIVE:

## Summary and Perspectives

The overall aim of this thesis was to determine the role played by miRNAs during caudal tail and spinal cord regeneration in the adult newt, and to determine which miRNAs may be either effectors or regulators of the retinoid signaling pathway in this regeneration-permissive system. I have demonstrated that many miRNAs are dysregulated not only in response to a SCI in the adult newt, but also in response to impaired retinoid signaling. Interestingly, not only do two of these miRNAs, miR-133a and miR-1, appear to target the retinoid receptor RARβ2, but inhibition of retinoid signaling with a RARβ selective antagonist also impairs the stage specific downregulation of these miRNAs during regeneration. These data are indicative of a negative feed back loop, albeit a potentially indirect one.

I also aimed to examine which miRNAs were affected by inhibiting RA synthesis during tail and caudal spinal cord regeneration. This analysis provided a significant list of miRNAs whose expression is dysregulated, and provides a foundation for future studies on their putative roles during caudal tail and spinal cord regeneration.

*MicroRNA dysregulation in response to caudal tail amputation.*

My first aim was to determine if any miRNAs were differentially expressed in the adult newt tail in response to amputation. I identified several miRNAs that were dysregulated in response to injury, including miR-124a, miR-132, miR-203 and miR-133a. Expression of miR-133a was localized to the ependymal cells surrounding the central canal of the spinal cord, and was high prior to injury but downregulated in response to tail amputation. RARβ2 was identified as a target of miR-133a, which is also localized to the ependymal cells in the adult newt spinal cord, and upregulated in

response to injury (Carter et al., 2011). Finally, mimic-based upregulation of miR-133a led to a decrease in RARβ2 levels and inhibition of regeneration. These data suggest that the downregulation of miR-133a in response to injury is required for the upregulation of RARβ2 for normal tail and caudal spinal cord regeneration.

*MicroRNAs 133a and 1 act downstream of RARβ2.*

My next specific aim was to determine which, if any, miRNAs were acting downstream of RARβ2-mediated retinoid signaling during tail regeneration. Eighteen miRNAs were significantly differentially expressed between the LE135 treatment and DMSO control groups, many with known roles in other systems that are associated with processes required for regeneration (eg., inflammation, apoptosis, proliferation and stem cell maintenance). Surprisingly, miR-133a was identified in this microarray, suggesting that it is not only acting upstream of RARβ2 signaling, but may also be a downstream effector of this signaling cascade.

At various timepoints during regeneration, miR-1, let-7c and miR-223 were localized to the nuclei of ependymal cells. miR-223 was transiently upregulated during the first 7 dpa, while miR-1 and let-7c were both downregulated in response to injury. RARβ2 was identified as a predicted target or miR-1, which was interesting as it belongs to the same genetic cluster as miR-133a (Georgantas et al., 2014; Wystub et al., 2013), and shares a very similar expression pattern during tail regeneration. RARβ2 was verified as a target of miR-1, and it would appear that together, miR-1 and miR-133a maintain basal levels of RARβ2 in the adult newt tail and spinal cord until they are downregulated in response to injury.

*Widespread differential miRNA expression in response to RA synthesis inhibition.*

While the above study determined which miRNAs were potentially acting as effectors downstream of RARβ2-mediated retinoid signaling, it is possible that other miRNAs may be involved in regulating other aspects of retinoid signaling. My next objective was to determine which miRNAs were differentially expressed in response to inhibition of retinoid synthesis during regeneration. To accomplish this, I again used a microarray, however this time I compared tissue treated with diethylaminobenzaldehyde (DEAB), a potent inhibitor of retinaldehyde dehydrogenase (RALDH2). Thus in this study, most, if not all RA synthesis should be inhibited with DEAB treatment. This microarray revealed 4,426 miRNAs that were differentially expressed in response to RA synthesis inhibition, and six were chosen for further profiling. Over the first 14 dpa, miR-23 expression levels did not change significantly temporally or between treatment and control groups. Conversely, miR-21 was significantly upregulated early after injury by 4 dpa compared to time 0, while both miR-205 and miR-25a were significantly upregulated by 7 dpa compared to time 0, but significantly downregulated from 7 to 14 dpa. Finally, both miR-149-3p and miR-206 were significantly downregulated at 14 dpa, for miR-149-3p relative to time 0, while miR-206 is significantly downregulated from day 7. Unraveling the role of each of these miRNAs during tail and spinal cord regeneration, and their association with the retinoid signaling pathway may provide a clearer picture of the complex molecular events underlying epimorphic regeneration in adult urodeles.

*Perspectives.*

It is clear that not only are miRNAs essential for tail and spinal cord regeneration in the newt, but that they play an integral role as effectors and mediators of retinoid

signaling through RARβ during this process. The precise role that each of these miRNAs play during regeneration is yet to be uncovered, and in reality may never be fully understood due to their pleiotropic effects on multiple targets. Will an understanding of the role of miRNAs as effectors of retinoid signaling be sufficient to develop therapies capable of overcoming the complex inhibitory environment that exists in the adult mammalian CNS after injury? This is not likely. It is unfortunately not one single cascade that contributes to the inhibitory environment for spinal cord regeneration in the mammal, but as discussed earlier in this thesis, a complex series of events that lead to secondary damage and scar formation.

In order to achieve functional regeneration after an SCI in mammals, several obstacles need to be overcome including inflammation, glial scar formation and non-permissive myelin associated inhibitors. A lack of retinoid signaling has been implicated in many of these events that leads to the inhibitory environment observed in mammals after SCI. For example, RA has been implicated as an anti-inflammatory factor, as treatment with high doses of RA in the rat lead to a reduction in the production of pro-inflammatory cytokines post-SCI (van Neerven et al., 2010). The expression of RARβ has been shown to overcome inhibition of neurite outgrowth by myelin-associated inhibitors (MAIs) both *in vitro* and *in vivo* by directly repressing the Nogo receptor Ngr (Agudo et al., 2010; Puttagunta and Di Giovanni, 2011). Furthermore, not only has our lab demonstrated that RA is capable of directing neurite outgrowth from adult newt spinal cord explants through RARβ-mediated signaling (Dmetrichuk et al., 2005), but that RARβ2 is necessary for the ependymal response after SCI in this species (Carter et al., 2011). Inhibition of RARβ leads to an inhibition in tail regeneration, and this may be due

to a lack of an ependymal response, as the ependymal tube fails to form (Carter et al., 2011).

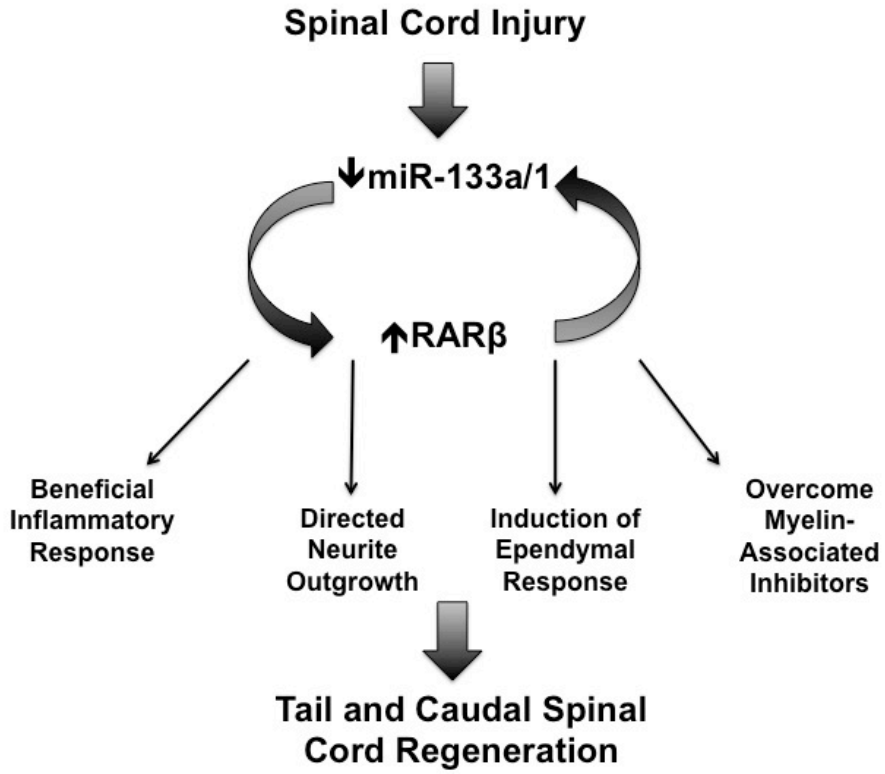


**Figure 5.1.** Proposed model of how miR-133a and miR-1 promote caudal spinal cord regeneration in adult newts. Downregulation of miR-133a and miR-1 in response to injury allows for an increase in RARβ2 expression. This forms a negative feedback loop to maintain this high RARβ2 expression, which may affect inflammation, activate the ependymal response, and direct neurite outgrowth despite an inhibitory environment.

Thus, the observed downregulation of both miR-133a and miR-1 may be required for the upregulation of RARβ during tail and caudal spinal cord regeneration in the adult newt. It is unknown what is regulating the miRNA dysregulation observed here in response to injury, but if this downregulation does not occur, RARβ expression may not be upregulated and tail regeneration would be inhibited (Fig. 5.1). It is possible that the increase in RARβ is required to initiate the ependymal response, where ependymal cells become reactive and proliferate to form the ependymal tube (Chernoff et al., 2003; Lee-

Liu et al., 2013). The increase in RARβ may also be involved in mediating inflammation to maintain a beneficial response rather than an inhibitory one. Finally, the upregulation of RARβ as a result of miR-1 and miR-133a downregulation may contribute to not only directed neurite outgrowth during regeneration, but also the intrinsic ability to overcome inhibitory MAIs.

Thus if a single, or cocktail, of miRNAs such as miR-1/133a was found to regulate retinoid signaling in a regenerative competent species its use as a treatment strategy in a regeneration-incompetent species might be warranted. Recent work by Diaz Quiroz et al., (2014) represented the first of its kind to examine and compare the pattern of expression and function of a miRNA, miR-125b, in the mammalian and urodele SCI models. These types of studies will be increasingly useful to develop miRNA-based therapeutic strategies for SCI. The possibility that miR-133a and miR-1 are targeting other genes in addition to RARβ cannot be ruled out, as miRNAs often have multiple targets. Overall these data provide a framework for future studies that elucidate the mechanisms that allow for functional spinal cord regeneration in this regeneration-competent urodele species.

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## Appendix

**Table 3.1.** Raw data from the microarray performed by LC Sciences (Houston, TX), comparing the signal intensities of LE135 or DMSO treated regenerates. Custom chips containing all known miRNAs from miRBase version 16.0 for *Danio rerio*, *Xenopus laevis* and *Xenopus tropicalis* were used.

| | | | Group 1 | Group 2 | |
|---|---|---|---|---|---|
| | | | DMSO & EtOH | LE135 | Log2 (G2/G1) |
| No. | Reporter Name | p-value | Mean | Mean | |
| 3 | dre-let-7c | 6.61E-03 | 633 | 505 | -0.33 |
| Following transcripts are statistically significant but have low signals (signal < 500) | | | | | |
| 168 | dre-miR-26a | 3.65E-07 | 92 | 190 | 1.05 |
| 249 | xla-miR-1306 | 1.03E-06 | 155 | 244 | 0.66 |
| 245 | dre-miR-92b | 7.69E-06 | 121 | 70 | -0.78 |

| | | | | | |
|---|---|---|---|---|---|
| 161 | dre-miR-223 | 1.16E-05 | 191 | 118 | -0.70 |
| 41 | dre-miR-133a | 1.10E-04 | 102 | 154 | 0.59 |
| 62 | dre-miR-145 | 1.79E-04 | 108 | 63 | -0.77 |
| 217 | dre-miR-462 | 2.54E-04 | 79 | 41 | -0.96 |
| 26 | dre-miR-125a | 2.63E-04 | 81 | 145 | 0.84 |
| 124 | dre-miR-205 | 2.73E-04 | 67 | 104 | 0.62 |
| 43 | dre-miR-133b | 6.56E-04 | 94 | 137 | 0.54 |
| 11 | dre-miR-1 | 1.08E-03 | 167 | 241 | 0.53 |
| 66 | dre-miR-150 | 3.29E-03 | 68 | 43 | -0.66 |
| 169 | dre-miR-26b | 3.75E-03 | 26 | 52 | 1.01 |
| 99 | dre-miR-193a | 5.06E-03 | 47 | 26 | -0.89 |
| 166 | dre-miR-24 | 5.65E-03 | 61 | 88 | 0.52 |
| 227 | dre-miR-727* | 7.09E-03 | 56 | 36 | -0.64 |
| 100 | dre-miR-193b | 9.03E-03 | 70 | 98 | 0.48 |

**Table 4.2.** A list of the miRNAs that were significantly different between DEAB and DMSO treatment groups in a microarray performed by LC Sciences (Houston, TX). Custom chips containing all known miRNAs from miRBase version 18.0 for Danio rerio, Xenopus laevis, Xenopus tropicalis, Homo sapien and Mus musculus were used. Red represents P<0.0001, yellow P<0.001 and blue P<0.01.

| Reporter Name | p-value |
|---|---|
| hsa-miR-1236-3p | 5.19E-05 |
| mmu-miR-6393 | 6.80E-05 |
| hsa-miR-3177-3p | 9.68E-05 |
| hsa-miR-6864-3p | 1.18E-04 |
| mmu-miR-485-3p | 1.25E-04 |
| mmu-miR-705 | 1.68E-04 |
| mmu-miR-6987-3p | 1.68E-04 |
| xla-miR-1b | 1.93E-04 |
| hsa-miR-3665 | 2.00E-04 |
| mmu-miR-466o-3p | 2.05E-04 |
| hsa-miR-4764-3p | 2.17E-04 |

| | |
|---|---|
| mmu-miR-7058-3p | 2.50E-04 |
| mmu-miR-467b-3p | 2.53E-04 |
| hsa-miR-1224-5p | 2.91E-04 |
| mmu-miR-6984-3p | 3.29E-04 |
| hsa-miR-4465 | 3.52E-04 |
| hsa-miR-6777-5p | 3.78E-04 |
| mmu-miR-1940 | 4.28E-04 |
| mmu-miR-7082-5p | 4.47E-04 |
| mmu-miR-6382 | 4.70E-04 |
| mmu-miR-7088-3p | 5.27E-04 |
| hsa-miR-6728-3p | 5.28E-04 |
| mmu-miR-7041-3p | 5.36E-04 |
| mmu-miR-6974-3p | 5.40E-04 |
| hsa-miR-6732-3p | 5.48E-04 |
| hsa-miR-1281 | 5.48E-04 |
| mmu-miR-5126 | 5.68E-04 |
| mmu-miR-8103 | 5.95E-04 |
| mmu-miR-8114 | 6.00E-04 |
| hsa-miR-3610 | 6.60E-04 |
| hsa-miR-6809-3p | 7.21E-04 |
| mmu-miR-3572-3p | 7.42E-04 |
| mmu-miR-669p-3p | 7.48E-04 |
| mmu-miR-7116-5p | 7.62E-04 |
| hsa-miR-4419b | 7.65E-04 |
| hsa-miR-7110-3p | 8.01E-04 |
| mmu-miR-6917-3p | 8.29E-04 |
| mmu-miR-7219-3p | 8.38E-04 |
| hsa-miR-5699-5p | 8.69E-04 |
| hsa-miR-1825 | 8.72E-04 |
| mmu-miR-7088-5p | 8.82E-04 |
| mmu-miR-709 | 9.29E-04 |
| mmu-miR-5107-5p | 9.89E-04 |
| xtr-miR-202-3p | 1.05E-03 |
| dre-miR-1 | 1.06E-03 |
| hsa-miR-6779-3p | 1.06E-03 |
| mmu-miR-7027-3p | 1.09E-03 |
| dre-miR-30c | 1.12E-03 |
| dre-let-7e | 1.15E-03 |
| mmu-miR-7070-3p | 1.16E-03 |
| mmu-miR-6939-5p | 1.17E-03 |
| hsa-miR-466 | 1.19E-03 |
| mmu-miR-3970 | 1.23E-03 |
| hsa-miR-4687-3p | 1.26E-03 |
| hsa-miR-6881-3p | 1.35E-03 |
| hsa-miR-4701-5p | 1.37E-03 |

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| mmu-miR-7036-3p | 1.42E-03 |
| mmu-miR-297a-3p | 1.43E-03 |
| mmu-miR-376b-3p | 1.44E-03 |
| dre-let-7d | 1.45E-03 |
| hsa-miR-6509-3p | 1.50E-03 |
| hsa-miR-6873-3p | 1.55E-03 |
| mmu-miR-6942-3p | 1.59E-03 |
| mmu-miR-341-5p | 1.62E-03 |
| mmu-miR-5110 | 1.73E-03 |
| mmu-miR-7001-3p | 1.74E-03 |
| hsa-miR-6780a-5p | 1.79E-03 |
| hsa-miR-4516 | 1.80E-03 |
| mmu-miR-1966-3p | 1.81E-03 |
| hsa-miR-7641 | 1.85E-03 |
| mmu-miR-877-3p | 1.86E-03 |
| mmu-miR-6368 | 1.87E-03 |
| mmu-miR-678 | 1.98E-03 |
| hsa-miR-1973 | 2.01E-03 |
| mmu-miR-6973b-3p | 2.03E-03 |
| dre-miR-125c | 2.19E-03 |
| mmu-miR-6961-3p | 2.19E-03 |
| hsa-miR-6750-3p | 2.20E-03 |
| mmu-miR-3071-5p | 2.24E-03 |
| hsa-miR-4298 | 2.26E-03 |
| dre-miR-130b | 2.32E-03 |
| mmu-miR-382-3p | 2.34E-03 |
| mmu-miR-6946-3p | 2.35E-03 |
| hsa-miR-6823-3p | 2.39E-03 |
| mmu-miR-3547-5p | 2.43E-03 |
| hsa-miR-6734-3p | 2.55E-03 |
| hsa-miR-1227-5p | 2.56E-03 |
| hsa-miR-5096 | 2.57E-03 |
| hsa-miR-449b-3p | 2.64E-03 |
| mmu-miR-6915-3p | 2.67E-03 |
| hsa-miR-6849-3p | 2.68E-03 |
| mmu-miR-6960-3p | 2.82E-03 |
| mmu-miR-5107-3p | 2.83E-03 |
| mmu-miR-6944-3p | 2.88E-03 |
| mmu-miR-7028-5p | 2.88E-03 |
| hsa-miR-485-3p | 2.92E-03 |
| mmu-miR-466g | 2.92E-03 |
| hsa-miR-6878-3p | 2.96E-03 |
| hsa-miR-6809-5p | 3.01E-03 |
| mmu-miR-467c-3p | 3.13E-03 |
| hsa-miR-6756-5p | 3.17E-03 |

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| mmu-miR-7658-3p | 3.24E-03 |
| hsa-miR-6775-5p | 3.25E-03 |
| xtr-miR-30c | 3.26E-03 |
| hsa-miR-3141 | 3.30E-03 |
| hsa-miR-483-3p | 3.36E-03 |
| mmu-miR-3968 | 3.60E-03 |
| mmu-miR-6978-3p | 3.65E-03 |
| xtr-miR-10b | 3.69E-03 |
| hsa-miR-4499 | 3.87E-03 |
| hsa-miR-4667-5p | 3.90E-03 |
| mmu-miR-7211-5p | 3.93E-03 |
| hsa-miR-6754-3p | 3.99E-03 |
| dre-miR-223 | 4.04E-03 |
| dre-miR-204 | 4.16E-03 |
| mmu-miR-1892 | 4.27E-03 |
| mmu-miR-8104 | 4.29E-03 |
| hsa-miR-6767-5p | 4.39E-03 |
| hsa-miR-6769a-5p | 4.55E-03 |
| mmu-miR-466m-3p | 4.57E-03 |
| hsa-miR-4281 | 4.81E-03 |
| hsa-miR-6778-5p | 4.84E-03 |
| hsa-miR-6830-3p | 4.90E-03 |
| hsa-miR-4443 | 4.97E-03 |
| hsa-miR-7856-5p | 5.08E-03 |
| mmu-miR-1895 | 5.27E-03 |
| mmu-miR-7056-3p | 5.28E-03 |
| dre-miR-26a | 5.29E-03 |
| hsa-miR-6891-5p | 5.36E-03 |
| dre-miR-214 | 5.47E-03 |
| hsa-miR-3682-3p | 5.53E-03 |
| hsa-miR-1224-3p | 5.77E-03 |
| hsa-miR-6816-3p | 5.78E-03 |
| mmu-miR-467a-3p | 5.81E-03 |
| mmu-miR-7017-3p | 5.90E-03 |
| mmu-miR-667-3p | 6.03E-03 |
| hsa-miR-6751-3p | 6.25E-03 |
| mmu-miR-196a-1-3p | 6.30E-03 |
| hsa-miR-4319 | 6.44E-03 |
| mmu-miR-1903 | 6.45E-03 |
| mmu-miR-149-3p | 6.45E-03 |
| hsa-miR-6832-3p | 6.60E-03 |
| hsa-miR-3197 | 6.61E-03 |
| mmu-miR-3569-5p | 6.74E-03 |
| hsa-miR-196b-3p | 6.74E-03 |
| mmu-miR-6967-5p | 6.81E-03 |

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| mmu-miR-669d-3p | 6.89E-03 |
| mmu-miR-5112 | 6.90E-03 |
| mmu-miR-483-5p | 6.91E-03 |
| xtr-miR-26 | 7.02E-03 |
| hsa-miR-6736-3p | 7.03E-03 |
| hsa-miR-6865-5p | 7.04E-03 |
| xtr-let-7i | 7.05E-03 |
| hsa-miR-23c | 7.23E-03 |
| hsa-miR-6831-3p | 7.32E-03 |
| mmu-miR-6914-3p | 7.75E-03 |
| mmu-let-7j | 7.82E-03 |
| dre-miR-10a-5p | 7.88E-03 |
| mmu-miR-7224-3p | 7.94E-03 |
| hsa-miR-6861-3p | 7.97E-03 |
| xtr-miR-98 | 7.99E-03 |
| hsa-miR-1260b | 8.35E-03 |
| mmu-miR-669d-2-3p | 8.45E-03 |
| dre-miR-125a | 8.57E-03 |
| hsa-miR-7106-5p | 8.76E-03 |
| hsa-miR-630 | 8.97E-03 |
| mmu-miR-467d-3p | 9.42E-03 |
| hsa-miR-4290 | 9.44E-03 |
| hsa-miR-6508-5p | 9.61E-03 |
| mmu-miR-466q | 9.66E-03 |
| hsa-miR-4690-5p | 9.84E-03 |